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STUDIES OF THE MOLECULAR RELATEDNESS OF H PLASMIDS USING RESTRICTION ENZYME DIGESTION AND SOUTHERN TRANSFER-DNA HYBRIDIZATIONS

by

MARY WHITELEY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Studies of the Molecular Relatedness of H Plasmids Using Restriction Enzyme Digestion and Southern Transfer-DNA Hybridization submitted by Mary Whiteley in partial fulfilment of the requirements for the degree of Master of Science.

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Abstract

The H incompatibility group of plasmids have played an important role in the mediation of antibiotic resistance in a number of medically important pathogens. They are characteristically large(>100Md), have a thermosensitive mode of transfer, and are unique amongst incompatibility groups in that they do not always show a high degree of DNA homology with other H plasmids. Based on batch DNA homology studies, three subgroups of H plasmids have been defined such that members of a given subgroup show a high degree of DNA homology with one another, but not with members of the other subgroups. These subgroups are called HI1, HI2, and HI3. A related group of plasmids designated HII have been described which are compatible with reference H plasmids, but share similar features to the HI group in that they are large and determine H pili.

This study concerns itself with a further examination of the molecular relationships which the H plasmids share through the use of restriction enzyme fingerprinting, and the Southern transfer-DNA hybridization technique. The isolation of H plasmid DNA suitable for such studies depends on the separation of this DNA from the bacterial chromosomal DNA. The isolation of large plasmid DNA has proven to be difficult as their physical properties more

closely resemble those of the chromosome than small plasmids. In this study, H plasmids were successfully isolated by three techniques. The merits and difficulties of each of these are discussed.

A representative member of each of the three HI plasmid subgroups was digested with a number of restriction enzymes. A diversity of restriction enzyme cleavage patterns was noted, and there appeared to be no bands common to all three subgroups. These subgroups did however show trends in the frequency of cutting with a given enzyme. Within a subgroup, plasmids had similar restriction enzyme cleavage patterns.

Southern transfer-DNA hybridization studies using a nick translated HI1 plasmid as a probe revealed that there was a small but distinct amount of homology among the members of the three HI subgroups. Of particular note was the hybridization of the HI1 plasmid to the HI3 plasmid digested with PstI, in which the homology observed was limited to three fragments in the 2-3kb size range. Within the HI1 subgroups, extensive DNA homology was noted in these hybridization experiments. An HII plasmid included in this study also demonstrated a small degree of homology with the HI1 plasmid.

Presumably, the restriction fragments showing DNA homology with the HI1 probe are responsible for some of the common phenotypic traits shared by the H plasmids.

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ABBREVIATIONS

BSA	Bovine serum albumin
CCC	Covalently closed circle
DNA	Deoxyribonucleic acid
EDTA	Disodium ethylene diamine tetraacetate.$2H_2O$
kb	kilobase
Md	Megadalton
mL	milliliter
µL	microliter
µg	microgram
M	moles
MW	molecular weight
O.D.	optical density
PVPD	polyvinylpyrrolidone
rpm	revolutions per minute
RNA	ribonucleic acid
SDS	Sodium dodecyl sulfate (also called sodium lauryl sulfate)
SSC	saline sodium citrate
Tris	tris(hydroxymethyl) aminomethane

CHAPTER I

INTRODUCTION

Bacteria often contain covalently closed double stranded DNA molecules which exist as distinct entities from the bacterial chromosome. These DNA molecules are called plasmids. Plasmids may code for a variety of genetic traits ranging from their own replication and maintenance to a number of metabolic functions such as resistance to antibiotics and heavy metals. Plasmids have also been shown to specify a number of properties that contribute to the pathogenicity of an organism such as toxin(Smith,1974) and hemolysin production (Smith & Halls,1967; Goebel & Schrempf, 1971) or the production of antigens associated with virulence(Orskov & Orskov,1966;Smith & Linggood, 1970).

Some plasmids are capable of promoting their own transfer to other bacteria through a process called conjugation. This allows the spread of plasmids to sensitive populations including inter-species transfer. Plasmids which are capable of transfer are called conjugative plasmids.

Plasmids coding for resistance to antibiotics(drug resistance plasmids)are known as R factors and are now found in many important pathogens including Salmonella sp.

Shigella, Pseudomonas, and more recently Haemophilus influenzae (Elwell, et al., 1975; Thorne and Farrar, 1975), and Neisseria gonorrhoeae (Seigel et al., 1978; Phillips, 1976). Plasmids are in a constant state of evolution in response to new drugs such as trimethoprim (Richards, 1978). Plasmids acquire resistance to antibiotics by transposition of DNA elements containing the genes for resistance (Hedges & Jacob, 1974; Bukhari et al., 1977). This constant evolution and spread of R factors has given rise to many complications in the effective treatment of both human and animal infections. An understanding of the genetics of plasmids may aid in the control of these elements.

1.1 Classification of Plasmids

Vast epidemiological data has been aquired about R factors since their discovery in Japan in 1958, and this has lead to a need to classify them into groups. The relatedness of R factors has been defined by several means, both phenotypic and genetic. Transferable antibiotic resistance plasmids were originally divided into two classes based on their effect on the transfer of the sex factor F of E. coli, a well characterized and studied plasmid (Watanabe & Fukasawa, 1962; Watanabe et al., 1962). Those plasmids which when present in the same cell as F inhibited the transfer of the F

factor were termed fi^+, while those which had no effect on the infectious transfer of F were termed fi^-. In these early studies on the classification of plasmids as fi^+ or fi^-, it was noted that two fi^+ factors were unable to coexist within the same cell(Watanabe et al., 1964). This property, the inability of related plasmids to stably coexist within a given host cell is termed incompatibility and has been further extended as a general classification scheme for plasmids. The plasmids examined in these earlier studies belonged to two incompatibility groups, one for fi^+, and one for fi^-. Further work to classify plasmids by incompatibility testing showed that within the fi^+ and fi^- classes plasmids could be divided further into incompatibility groups (Chabbert et al., 1972;Hedges & Datta, 1972; Hedges & Datta, 1973). That is fi^+ and fi^- plasmids were compatible but within each class, pairs of plasmids were incompatible. Incompatibility and compatibility relationships are found in all naturally occurring plasmids. Using this scheme, some 26 groups have been defined for R plasmids from Enterobacteriaceae . A detailed list of reference plasmids from these incompatibility groups is given in the book DNA Insertion Elements, Plasmids and Episomes, Cold Spring Harbor, New York, 1977. Table 1 lists some of the incompatibility groups and reference plasmids from these groups.

To test the incompatibility of a given pair of plasmids, a plasmid(incoming plasmid) is introduced

Table 1

Plasmid Incompatibility Groups and Their Hosts

Inc group	Plasmid Example	Resistance and Other markers[a]	Original Bacterial Host
A	RA1	Su,Tc	*Aeromonas liquefaciens*
C	R40a	Ap,Km,Su	*Pseudomonas aeruginosa*
FI	R455-2	Ap,Cm,Km,Su,Sm	*Proteus morganii*
FII	R1	Ap,Cm,Km,Su,Sm	*Salmonella paratyphi*
FIII	ColB-K98	ColB	*Escherichia coli*
FIV	R124	Tc,Hsp1	*Salmonella typhimurium*
HI1	R27	Tc	*Salmonella typhimurium*
HI2	R478	Cm,Km,Tc,Te	*Serratia marcescens*
HI3	MIP233	Te,suc	*Salmonella Ohio*
HII	pHH1508a	Sp,Tp,Te	*Klebsiella aerogenes*
I	R144	Km,Tc,ColB	*Salmonella typhimurium*
J	R391	Km,Sm,Hg	*Proteus rettgeri*
L	R831	Km,Sm	*Serratia marcescens*
M	R466b	Sm,Tc	*Proteus morganii*
N	N3	Sp,Su,Tc,Hg,HspII	*Shigella flexneri*
O	R16	Ap,Sm,Tc,Su	*Shigella dysenteriae*
P	RP4	Ap,Km,Tc,	*Pseudomonas aeruginosa*
T	Rts1	Km	*Proteus mirabilis*
W	Sa	Cm,Km,Sm,Su	*Shigella flexneri*

[a]Ap;ampicillin;Cm,chloramphenicol;Sm,streptomycin;Sp, spectinomycin;Su,sulfonamides;Tc,tetracycline;Hg mercuric choride;Km, kanamycin;Te potassium tellurite ;Tp, trimethoprim;suc;sucrose fermentation;ColB,colicin B ; Hsp; host specificity

into a cell containing a plasmid(resident plasmid). Selection is made for the incoming plasmid, and clones from this are then tested for the loss of one of these plasmids. Usually the resident plasmid is lost in such a test. Testing of plasmids in such a manner usually gives unambiguous results, but difficulties may arise from unstability of the plasmids in a given host.

1.2 Correlation of incompatibility grouping with other plasmid functions

A) Production of pili

Conjugative plasmids code for pili or sex fimbriae which are morphologically distinct filaments extruded from bacterial cells. It has been shown that plasmids within a given incompatibility group determine morphologically and serologically similar pili(Bradley, 1980). As well there are a group of "male specific phages"which recognize these plasmid encoded pili, but not common or Type I pili. Examples of such phages are MS2 which absorb to the sides of F pili(Bradley & Meynell,1978), PRR1 which lyses bacteria carrying Inc P pili(Olsen & Thomas,1973;Bradley, 1976) and If1 which is specific for Iα pili.

B) Entry Exclusion

One further plasmid encoded function which shows

a relationship to incompatibility is entry exclusion. Entry exclusion is defined as the ability of a plasmid to inhibit the entry of another related plasmid into the same host cell. The site of action of entry exclusion is at the level of the cell surface. Not all conjugative plasmids show entry exclusion, but when they do it is usually against members of the same incompatibility group.

C. Resistance determinants

Resistance determinants of a plasmid show little correlation with incompatibility grouping. This is probably true since many of the genes carried by plasmids, including antibiotic resistance, are located on transposable elements and the exchange of genetic material amongst related plasmids occurs rapidly.

D. Molecular relatedness of plasmids grouped by incompatibility testing

The amount of DNA sequence homology amongst incompatibility groups has been examined by DNA-DNA hybridization techniques. This involves the denaturation of the DNA species to be examined to single stranded form. Homologous DNA from the two species will reanneal in such a reaction. The amount of homology is monitored by radioactively labelling one of the species involved in the hybridization

reaction. The percent of DNA homology amongst plasmid incompatibility groups was determined by one of two methods of DNA hybridization. Grindley et al, 1973, used a batch hybridization method using hydroxyapatite(HA) to separate single stranded DNA from double stranded DNA. Essentially, ^{3}H labelled and unlabelled plasmid DNA preparations were sheared to give an average size of about 0.2Md(300 base pairs). These were then heat denatured at 100°C, and were mixed and allowed to renature. A 2000-7000 fold excess of unlabelled DNA was used in the reaction to prevent renaturation of the labelled plasmid with itself. Following renaturation, the reassociated DNA was separated from single stranded DNA by the addition of the reaction mixture to HA equilibrated with 0.14M phosphate buffer. The reassociated DNA(double stranded) binds to the HA, while single stranded DNA remains in the supernatant. The HA is removed by centrifugation and the double stranded DNA is then eluted from this with 0.4M phosphate buffer. The number of counts represented as single and double stranded DNA is then determined. As a control of reassociation of labelled DNA with itself, the unlabelled DNA is replaced with E. coli chromosomal DNA in a seperate reaction. The percent of DNA homology is then estimated as the proportion of counts of reassociated DNA (double

stranded) to the total number of counts(double stranded plus single stranded). Of course, the calculation of this value takes into account the percentage of reassociation in the control mixture. Roussel and Chabbert, 1978, quantitated DNA sequence homology with hybridizations performed on nitrocellulose filters. In order to do this, 4 kinds of filters were prepared;one was prepared with E. coli chromosomal DNA(R^-hom filter); one with homologous plasmid DNA and chromosomal DNA (R^+ hom filter); one with heterologous plasmid DNA and chromosomal DNA(R^+ het filter), and one with no DNA. The DNA added to these filters was first denatured with alkali, and then fixed to the filters with heat(80°C) under vacuum. The filters were then probed with a preparation of ^3H labelled alkaline denatured plasmid DNA. The probe was in 50% w/v formamide and 2XSSC buffer. An aliquot of this was placed on each of the filters, and hybridization was performed under paraffin oil overnight. Following hybridization, the unbound label (DNA which did not reanneal) was washed off. Using this method, the percent of homology was determined as :

$$\frac{\text{counts bound to } R^+\text{het filter-counts bound to } R^-\text{filter}}{\text{counts bound to } R^+\text{hom filter-counts bound to } R^-\text{filter}} \times 100$$

Again an excess of unlabelled DNA was used in this reaction.

The results from these studies revealed three general patterns of DNA relatedness.

1. Plasmids within a given incompatibility group that show a high degree of homology with each other(up to 75%)

2. Plasmids of a given incompatibility group that show significant homology with several incompatibility groups. For example, members of the O incompatibility group demonstrated 20-35% homology with members of the I group. They did not specify I pili.

3. Members of the H incompatibility group did not always show a high degree of DNA homology with some other members of the H group.

In general, plasmids of the same incompatibility group have similar molecular weights.

Other molecular techniques including restriction enzyme fingerprinting(Thompson et al, 1974), and heteroduplex mapping (Sharp et al., 1973) have been used to study the relatedness of plasmids.

1.3 The nature of incompatibility

The molecular basis of incompatibility is not yet understood, although certain aspects are clear.

Plasmids have the ability of being maintained at a constant level within a given host cell(copy number) and are stably inherited by daughter cells. Copy number of plasmids ranges from 1 to about 50 copies per genome. The implication of this is that plasmid replication is strictly regulated. Plasmids with low copy number must therefore be under stringent regulation ensuring that each daughter cell receives one copy of the plasmid. It is thought that incompatibility is due to the processes involved with interference of DNA replication or the orderly partitioning of these elements to daughter cells (see Novik & Hoppensteadt, 1978, for a review).

Classical genetic studies of incompatibility have been hindered by the failure to obtain inc^- mutants. This would indicate that incompatibility is a complex genetic system comprised of several gene loci. With the advent of recombinant DNA technology and cloning the molecular basis of incompatibility has proven this to be the case. One system which has given insights into incompatibility genes has been from cloned segments of a plasmid called mini-F(Timmis et al.,1975;Lovett & Helinski, 1976), which is a derivative of the E.coli sex factor F. The F factor is about 94.5kb in size. It is a member of the FI incompatibility group and is a low copy number plasmid(1-2 copies/genome). It is

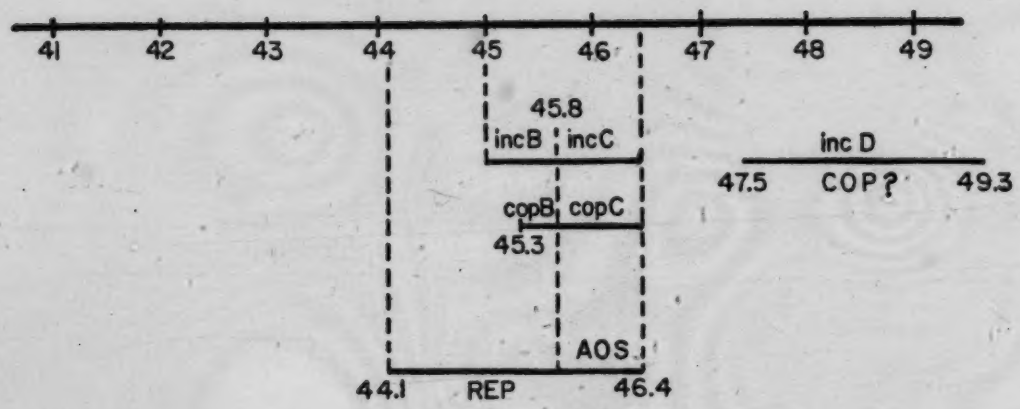
cut into 19 fragments by the restriction enzyme EcoRI. Mini-F is derived from fragments f5 of this digest and contains the sequences necessary for normal maintenance of F(40.3-49.3kb on the F map). Within this region, two replication origins have been located. One designated OriV is the primary origin of replication and it is detected by its sensitivity to acridine orange(Eichenlaub et al.,1977) The other , OriS, which is resistant to acridine orange treatment, functions when OriV has been deleted(Figurski, et al.,1978). The regions expressing incompatibility are also found in this region and are incB, incC, and incD(Manis & Kline, 1978; Kline & Lane, 1980; Lane, 1981). These regions have been mapped by deletion derivatives of mini-F and transposition mutagenesis, followed by cloning experiments for further characterization of these regions. The incompatibility loci are found between 45.1 and 49.2kb on the F map. A summary of the genes in this region is given in figure 1. The region 45.0-45.8 contains the incB function and mutants in this region obtained by chemical mutagenesis effect the copy number of the plasmid, although incompatibility is still expressed (Kahn et al.,1979). The locus in this region affecting copy number is called copB. Mutagenesis with the transposon Tn3 in the region 45.8-46.5 containing

the incC locus also affects copy number and the incC phenotype(Kahn et al., 1979) The region affecting copy number is copC. OriV is also in this region. Mutagenesis of the incD region does not result in any profound effect on copy number(Kahn et al., 1979). When these incompatibility regions have been cloned onto a plasmid normally compatible with F, pSC101, the recombinant plasmid expresses incompatibility with F. One feature which distinguishes incD with incB or C is that the cloned incD genes express incompatibility not only with F derivatives but also with R386, another FI plasmid, whereas cloned incB and C genes are compatible with R386(Kline, 1979). The DNA sequence of the region containing the incC region has recently been determined in order to discern if this region codes for a protein which interferes with F replication(Tolun & Helinski, 1981). The results of these experiments indicate that it does not code for such a protein, but the salient feature of this region is the existence of five 22 base pair repeats. When these repeats are cloned onto pACYC184, they express incompatibility. The exact role of these repeats is not clear with regards to incompatibility, although they may play a role in the initiation of replication. Recently a further region of F expressing incompatibility has

Fig. 1

A map of the replication, incompatibility, copy number and acridine orange sensitivity genes of F.

(Kline *et al.* 1981)


41
42
43
44
45
46
47
48
49
45.8
incB
incC
inc D
47.5
COP?
49.3
copB
copC
45.3
AOS
44.1
REP
46.4

been cloned from the EcoRI fragment f7(32.8-40.3 on the F map). It has been called incE(Bergquist et al.,1982).

Recent results using these cloned incompatibility regions as probes in southern transfer DNA hybridization experiments point out that not all the plasmids designated as FI incompatibility group plasmids share all these sequences(Bergquist et al., 1982). That is to say that complete homology of all the incompatibility regions is not a necessary requirement for the classification of a plamid as FI. Although these incompatibility sequences have been identified to date, there is no encompassing model to explain the phenomemon of incompatibility.

1.4 Modes of pilus synthesis of plasmids

The genes for conjugal transfer of F and F-like plasmids have been well characterized. The genes for transfer of the sex factor F are located in the 60. -93.2 kb region of the F map. Nineteen genes have been mapped in this region which are involved in pilus synthesis and control of gene expression(see Willets and Skurray,1980 for a review). Control of expression of genes in the transfer region operate at two levels. The product of a gene called traJ provides positive control of expression, while the expression of traJ is controlled

by the finOP system. The products of the finO and finP genes interact to prevent the expression of the traJ gene. In the absence of the traJ gene product, synthesis of all the other transfer operon products is prevented. Also within the transfer operon are the genes for entry exclusion, traS and traT. Point mutations in traS are efficient for transfer, while the gene product of the traT gene is an outer membrane protein associated with the expression of entry exclusion. The result of the control of pilus synthesis in such a manner is that not all cells in the population produce pili. Such a mode of pilus synthesis is termed repressed. Transfer of repressed plasmids occurs by the transient expression of the transfer genes in a small percent of the cells harboring plasmids. The sex factor F is $finO^-$, and $finP^+$, and thus produces pili constitutively, and is termed derepressed for transfer. Infectious transfer of this plasmid occurs with a high mating frequency (mating frequency being defined as the number of transconjugants per donor cell).

1.5 The H plasmid incompatibility group

Many medically important plasmids belong to the H incompatibility group . These plasmids will be the subject of investigation in the experiments presented in this thesis. H plasmids

were first described in an outbreak of typhoid fever in Mexico in 1972(Grindley et al ., 1972). Although chloramphenicol resistance had been previously described in individual strains of S. typhi, this was the first incidence of an epidemic involving resistant strains. This was of clinical significance since chloramphenicol treatment had proven to be extremely efficacious in the treatment of typhoid fever. The strains isolated from patients in this outbreak were resistant to tetracycline, chloramphenicol, streptomycin, spectinomycin, and sulfamethoxazole. H group plasmids have subsequently been reported in outbreaks of typhoid fever throughout the world(Anderson, 1975;Anderson & Smith, 1972;Datta & Olarte, 1974).

As a group, H plasmids have several distinguishing features. They are large(>100Md in size),and exhibit a thermosensitive mode of transfer(Smith, 1974). In addition they determine morphologically and serologically similar pili(thick and flexible)(Bradley, 1980). H plasmids are repressed for transfer. The plasmids in this group are classified into three subgroups based on DNA homology studies(Roussel and Chabbert, 1978)

Two subgroups of H plasmids, H1 and H2 were originally designated on their incompatibility reaction with F factors in the autonomous state(Smith et al., 1973). H1 plasmids were incompatible with F factors

while H2 plasmids were compatible with F factors. Furthermore, H1 plasmids like the one involved in the outbreak of typhoid fever in Mexico, have been primarily associated with chloramphenicol resistance in S. typhi, and while H2 plasmids were originally identified in strains of Salmonella, they have also been found to mediate antibiotic resistance in other Enterobacteriaceae, , including Shigella flexneri(Taylor & Grant, 1977a),Serratia marcescens(Taylor & Grant, 1977b) Citrobacter freundii(Taylor & Summers, 1979), and Klebsiella pneumoniae(Smith et al.,1978). Members of the H2 subgroup also encode for bacteriophage inhibition: that is H2 plasmid-containing strains inhibit the development of a number of double stranded bacteriophage including λ,T1, T5, and T7(Taylor & Grant, 1978). Resistance genes for potassium tellurite are also commonly found on H2 plasmids(Taylor & Summers,1979). Members of the H1 and H2 subgroups show entry exclusion with each other. Members within a given subgroup have a higher index of entry exclusion with each other than with members of the other subgroup(Taylor & Grant,1977b.) Another interesting feature of the H plasmids is that some of them can be eliminated at high temperatures (Smith et al.,1978)

DNA-DNA hybridization studies of members of the H plasmid incompatibility group revealed that there

is little homology between members of the H1 and H2 subgroups,although H1 subgroup members show a high degree of homology with each other as do H2 subgroup plasmids. Based on work by Roussel and Chabbert, 1978, a third subgroup comprised of one member , MIP233, was designated which was incompatible with H1 and H2 plasmids, but showed little DNA homology with either of these two subgroups. This plasmid confers resistance to potassium tellurite , encodes sucrose fermentation, and specifies H pili.

Recently plasmids which show a relationship with H plasmids have been described(Bradley et al., 1982). These plasmids which were isolated from Klebsiella aerogenes, are large and determine conjugative pili of the H type. They are incompatible with each other, and show entry exclusion with each other. They also determine resistance to potassium tellurite. Unlike the H plasmids they are derepressed for transfer, and their mode of transfer is not thermosensitive. In incompatibility tests, they show a stable coexistence with members of the H1,H2, and H3 subgroups. The designation incHII has been proposed for these plasmids, while H plasmids are now called HI1, HI2, and HI3. This designation is similar to the FI and FII plasmids which are compatible but are related by antigenically similar pili.

The lack of homology amongst the H plasmid subgroups lends itself to studying and identifying the genes which they share in common. Presumably the genes responsible for incompatibility are amongst these. As mentioned earlier with the studies of the FI plasmids, complete homology of all the incompatibility genes is not a necessary requirement for a plasmid to be classified as FI. Complete homology of the H plasmids with respect to their incompatibility genes is already ruled out because of the differential incompatibility of the HI plasmids with the F factor. A small amount of homology between HI1 plasmids and F has been found on a 5Md EcoRI fragment of incHI1 plasmids(Bergquist, personal communication). This fragment may contain some of the sequences for FI incompatibility described in section 1.3. The H plasmid system may reveal incompatibility sequences unique for each of the subgroups as well as common incompatibility sequences. Other common genes may include those responsible for transfer, pilus production and entry exclusion.

The work in this study is concerned with the molecular characterization of the H plasmids, and the identification of the common sequences shared by these plasmids by Southern transfer DNA hybridization techniques. This involves the transfer of restriction endonuclease fragments to nitrocellulose followed

by probing of these fragments with a ^{32}P-labelled H plasmid. These plasmids represent an interesting group and the lack of DNA homology amongst the subgroups may indicate that they have evolved separately. They may provide valuable tools for increasing our understanding of the genetics of plasmids.

CHAPTER II

METHODS AND MATERIALS

2.1 Culture conditions and media

Prior to isolation , plasmid containing strains were streaked out on antibiotic plates containing a suitable drug to select for the presence of the plasmid. Antibiotic plates were prepared in MacConkey agar (Difco), except for potassium tellurite, which was prepared in Brain Heart Infusion agar(Difco). The final concentrations of the antibiotics used are listed in Table 2, with the appropriate abbreviations for the antibiotics. Plasmids were isolated from broth cultures. The broth used for isolation was a rich minimal media(RM),(Murialdo & Siminovitch, 1971). The components of RM are detailed in Table 3.

2.2 Bacterial Strains and Plasmids

The plasmids and their relevant properties are listed in Table 4. All plasmids were harbored in derivatives of *E. coli* K-12.

Table 2

Antibiotics -Concentrations and Abbreviations

Antibiotic	final concentration ug/mL	Abbreviation
Ampicillin	24	Ap
Chloramphenicol	16	Cm
Kanamycin	8	Km
Tetracycline	8	Tc
Streptomycin	10	Sm
Sulfamethoxazole	1000	Su
Spectinomycin	40	Sp
Potassium tellurite	$2.5X10^{-5}M$	Te

Table 3.

Rich minimal media(RM)

25XRM salts

per200mL

NH_4Cl	5.0g
$MgSO_4.7H_20$	1.23g
KCl	7.5g.
glycerol	8.0mL.
.01M $FeCl_3$	1.5mL

Phosphate Buffer

per 940mL

Na_2HPO_4	7g.
KH_2PO_4	3g.

per 1 liter of RM

phosphate buffer	940mL
25X RM salts	40mL
20% glucose	6mL
10% casamino acids	20mL
100mM $CaCl_2$	1mL

Table 4 . Plasmids and Their Relevant Properties

Plasmid Designation	Resistance Pattern	Original Host	Place of origin and date	Reference
a) incHI1 plasmids				
pRG1251	Ap,Cm,Su,Sp,Tc	S.typhi	Thailand,1972	Taylor & Grant,1977a
pRG1271	Su,Tc,Cm,Sm	S.typhi	Mexico ,1972	D.E. Taylor,unpublished
pRG1284	Cm,Sm/Sp,Su,Tc	S.typhi	Vietnam, 1972	D.E. Taylor,unpublished
R27	Tc	Salmonella typhimurium	England,1961	Grindley et al,1972
TP123	Cm,Sm,Su,Tc	S.typhi	Mexico,1972	Anderson et al.,1972
b) incHI2 plasmids				
R476b	Tc,Sm,Su	Ser.marcescens	U.S.A.,1969	Taylor and Levine,1980
R826	Ap,Cm,Gm,Km	Ser.marcescens	France,1974	Taylor and Levine,1980
TP116	Cm,Sm,Su,Te	S.typhi	Spain,1969	Grindley et al.,1972
MIP235	Cm,Sm,Su,Te	Salmonella oranienburg	Brazil,1973	Roussel & Chabbert,1978
R478	Cm,Km,Tc,Te	Ser.marcescens	U.S.A.,1969	Taylor & Grant,1977b
c) incHI3 plasmids				
MIP233	Te,Scr	Salmonella ohio	England,1972	Roussel & Chabbert,1978
d) HII plasmids				
pHH1508a	Sm,Tp,Te	Klebsiella pneumoniae	England,1982	Bradley et al.,1982

2.3 Isolation of bacteriophage lambda DNA

Bacteriophage λ DNA was prepared from a λcI857 lysogen obtained from H. Murialdo. Cells were grown in 600mL of L-Broth(1% tryptone, 1% sodium chloride, and 0.5% yeast extract, pH7.2) to an $O.D._{600}$ of 0.6 at 30°C. The phage were then induced at 42°C for 15 minutes, followed by growth of the cells at 37°C for 90 minutes. The cells were then harvested by centrifugation at 8,500 rpm for 10 minutes in a Beckman JA-14 rotor. Lysis of the cells was accomplished by the addition of chloroform(5 mL) and a few flakes of DNAse (Worthington). The lysate was incubated at 37°C for 10 minutes. The phage were then purified in CsCl density gradients by the addition of 0.75g CsCl/mL of lysate. The gradients were spun in a SW40 rotor at 29,000 rpm for 36 hours. The phage band was extracted from the gradient with a 21 gauge needle and dialyzed against λ diluent(10mM $MgSO_4$, 10mM Tris, pH 7.4). The DNA was then extracted from the phage by the addition of EDTA to 0.01M, and SDS to 0.3% final. Proteinase K (Boehringer Mannheim) was added to 100 µg/mL and incubated at 37°C for one hour. The DNA was then extracted twice with Tris-equilibrated phenol, and then dialyzed against 8mM Tris, 10mM NaCl, and 0.1mM EDTA, pH 7.6. Following dialysis, the DNA was stored at 4°C.

2.4 Isolation of H plasmid DNA

A) Method I; Sarkosyl lysate method

H plasmid DNA was prepared from sarkosyl lysates as previously described(Lin & Kado, 1977), with the following modifications. Cultures were grown in 500mL of a rich minimal medium(RM), to an $O.D._{600}$ of 0.4-0.6. The cells were then harvested by centrifugation at 8,500 rpm for 10 minutes, and were resuspended in 20mL of sterile water and pelleted again in a JA20 rotor at 9,000 rpm for 10 minutes. The cells were then resuspended into 40 mL of solution A(0.1M NaCl, 0.02M Na_2EDTA, 20% sucrose,pH 8.0), and 40 mg of lysozyme(Boehringer Mannheim) were added. After incubation on ice for 10 minutes, the mixture was divided into 4 tubes(10mL/tube, and to each tube 10 mL of solution B was added (1% sarkosyl NL-97(Geigy), 0.01M Na_2EDTA, 0.5M NaCl, pH8.0). The solutions were mixed by inversion, and were allowed to stand at room temperature for 20 minutes. The cleared lysates were then treated with RNAse(Boehringer Mannheim) at a final concentration of 50µg/mL for 20 minutes at 25°C. Pronase was then added to a final concentration of 200µg/mL to the HI1 plasmid lysates and proteinase K was added to the HI2 or HI3 lysates to a final concentration of 150µg/mL. Protease treatments were for 25 minutes at 37°C. Pronase and proteinase

K were obtained from Boehringer Mannheim.

B) Isopynic CsCl-ethidium bromide Density Gradient Centrifugation

1gm/cc of CsCl was added to the sarkosyl lysates which were then transferred to 8 heat sealable Beckman polyallomer tubes. 0.15mL of a 10mg/mL solution of ethidium bromide was added to the tubes in the dark. The tubes were filled with a CsCl solution of 1gm/cc in 8mM Tris, 10mM NaCl, and 0.1M EDTA before sealing. Samples were centrifuged in a Ti-75 rotor for 24 hours at 55,000 rpm. The chromosomal bands(upper bands) were removed first through the top of the tubes with a pasteur pipette. Then the plasmid bands were removed with a syringe with an 18 gauge needle through the sides of the tubes. The plasmid DNA was pooled and rebanded in CsCl as before. The rebanded plasmid DNA was extracted several times with isoamyl alcohol, and dialyzed exhaustively against a buffer containing 10mM Tris, 5mM NaCl, and 1mM EDTA, pH 7.6.

C) Method II: Alkaline denaturation method:Kado and Lui.

A scaled up version of the alkaline denaturation method described by Kado and Liu (1981) was used with the following modifications. Cells were grown in 500mL of RM, to an O.D. $_{600}$ of 0.4-0.6, and were then harvested

by centrifugation in a Beckman JA-14 rotor for 20 minutes at 7,500 rpm. The cells were resuspended in 15mL of TEA buffer(40mM Tris acetate, 2mM EDTA, pH 8.0), and repelleted in a JA-20 rotor for 12 minutes at 9,000 rpm. The pellet was resuspended in 10 mL of TEA buffer, and 5mL of lysis buffer(3% SDS, 50mM Tris,pH 12.6), and heated at 60°C in a water bath for one hour. The lysate was then extracted once with Tris equilibrated phenol, and once with phenol:chloroform(50:50 ratio). The aqueous layer was treated with RNAse(Boehringer Mannheim) at a final concentration of 50µg/mL for 20 minutes at 37°C, followed by treatment with pronase(200µg/mL final) or proteinase K(150µg/mL final) at 37°C for an additional 20 minutes. The DNA was then ethanol precipitated by the addition of NaCl to 0.3M final, and 2.5 volumes of cold ethanol. The solution was then placed at -20°C overnight and the next day was spun at 10,500 rpm for 30 minutes. The pellet was resuspended in 5 mL of 8mM Tris, 10mM NaCl,and 0.1mM EDTA,pH7.6. The plasmid was further purified by CsCl ethidium bromide density gradients with the addition of 1gm/mL CsCl as described in section 2.4(B), using a single banding step.

D) Method III:Alkaline denaturation method:Birnboim and Doly

A scaled up version of the alkaline denaturation

method described by Birnboim and Doly(1979), was used to isolate H plasmid DNA as follows. Cells were grown in 150mL of RM overnight, pelleted in a Beckman JA-14 rotor at 8,500 rpm for 10 minutes and resuspended in 2.5mL of solution I(50mM glucose, 10mM EDTA, 0.25M Tris , pH8.0, and 2mg/mL lysozyme(made fresh daily)). The cells were then incubated on ice for 30 minutes. Five mL of solution II was added(0.2N NaOH, 1%SDS), and the preparation was incubated for another 5 minutes on ice. A volume of 3.75mL of solution III(3M sodium acetate, pH4.8) was then added and the lysate was incubated on ice for one hour, followed by centrifugation for 15 minutes at 12,500 rpm . The supernatant was ethanol precipitated by the addition of 2 volumes of cold ethanol, and kept at -20°C overnight. On the following day, the DNA was recovered by centrifugation at 10,500 rpm for 10 minutes. The pellet was then resuspended in 5 mL of 50mM Tris, 10mM EDTA, and treated with RNAse at a final concentration of 50µg/mL for 20 minutes at 37°C. The plasmid DNA was purified in CsCl ethidium bromide density gradients as described in section 2.4(B), using a single banding step.

2.5 Determination of DNA concentration

DNA concentrations were determined by absorbance at 260 nm with a Beckman DU 8 spectrometer. When

determinations were required, the concentraion of DNA was measured by the fluorescence method (Morgan et al. 1979) using a Turnbull fluorimeter.

2.6 Restriction Enzyme Digestions

Restriction enzyme digestions were performed according to the conditions specified by Davis, Botstein and Roth (1980). Approximately 0.5-1.0 µg of H plasmid DNA was digested for 80 minutes at 37°C. The digestion was stopped by the addition of EDTA to 12mM final concentration and 1/5 volumes of bromphenol dye mix (0.075% bromphenol blue (Biorad), 48% sucrose). The reaction was then heated at 71°C for ten minutes. Double digestions were performed using a buffer suitable for both enzymes as determined empirically. The enzymes used, and the conditions for digestion are listed in Table 5.

Phage λ DNA digested with either EcoRI, or HindIII was used as molecular weight standards. Approximately 0.3µg of λ DNA was digested as a control. The molecular weights of these DNA standards are given in Table 6.

2.7 Agarose Gel Electrophoresis

Restriction enzyme digestions were electrophoresed on a horizontal gel apparatus at 50V for 16-18 hours.

Table 5

Restriction Enzymes and Digestion Conditions

Enzyme(s)	Buffer*	Temp(°C)	Source
AccI	med	37	BRL[a]
AvaI	med	37	BRL
AvaII	med	37	BRL
BamHI	med	37	BMC[b]
BglII	med	37	BMC
EcoRI	high	37	BMC
HindIII	med	37	BMC
HpaI	low	37	BMC
KpnI	low	37	BRL
PstI	med	30	BMC
PvuII	med	37	BRL
SalI	high	37	BMC
Sau96I	med	37	BRL
XbaI	high	37	BMC
PstI/SalI	high	37	
PstI/XbaI	high	37	

*buffer conditions are as described by Davis, Botstein and Roth;high,med, and low refer to salt conditions in these buffers

a Bethesda research laboratories
b Boehringer Mannheim Canada

Table 6

Sizes of the restriction fragments of phage λDNA[a]

Fragment	EcoRI	HindIII
A	21.8	23.7
B	7.52	9.46
C	5.93	6.61
D	5.54	4.26
E	4.80	2.26
F	3.41	1.98
G		.58

a Sizes of the fragments is given in kilobase pairs as determined by Phillippsen et al. 1978. Values given are for λcI857

The buffer used for electrophoresis was 89mM Tris, 89mM boric acid, 3.4mM EDTA,pH8.3. The concentration of agarose(Seakem) varied from 0.5%-0.8%. The gels were photographed under ultravioled transillumination with a Pentax 35mm camera with a red filter using Kodak Tri-X film.

2.8 Computer Analysis of Restriction digests:Estimation of fragment sizes

The sizes of the DNA fragments from the restriction digests were obtained from a computer program(Schaffer & Sederoff,1981) which was adapted for use on the computer at the University of Alberta. The program is based on a relationship described by Southern(1979), and states that the reciprocal of mobility versus fragment length is linear. This program uses a least squares analysis of this relationship. The standard curve is generated from the known sizes of λ DNA digested with EcoRI or HindIII . A listing of the program is given in Appendix 1.

2.9 Electron Microscopy of DNA

DNA preparations were diluted to 1-5μg/mL, and were mounted on 200 mesh copper grids coated with parlodion . Samples were spread by the formamide technique of Davis et al.(1971). The grids were shadowed with platinim/paladium, and were examined

with a Phillips 300 electron microscope. When pBR322 was added as an internal standard, it was added to the DNA preparations at a final concentration of 0.1-0.3µg/mL .

2.10 Southern transfers

Plasmid DNA was digested as described in section 2.6, and transferred to nitrocellulose as described by Southern(1975). Nitrocellulose was obtained from Schleicher and Schuell(BA85)

2.11 NIcked Translation

Approximately 0.5µg of HI1 plasmid DNA(pRG1251) was labelled in a nicked translation reaction as described by Rigby et al., 1977. 100µCi of α-^{32}P-dCTP in tricine(NEN) was used in a reaction volume of 120µL. The reaction conditions were as follows: 50mM potassium phosphate(pH 7.4), 5mM $MgCl_2$, 10µM dTTP,dATP, and dGTP, and 50µg/mL BSA. Prior to the addition of DNA polymerase I, the DNA was treated with DNAseI (1ng) for one minute at room temperature. 2.5 units of PolI(Boehringer Mannheim) was added, and the reaction was incubated at 14°C. The reaction was terminated by the addition of 500 µL of stop buffer(100µg/mL herring sperm DNA in HENS buffer), and heating the reaction at 65°C. Unincorporated label was separated from the nicked translated products

by passing the reaction over a 5mL sephadex G50 fine column equilibrated with HENS buffer(10mM HEPES,pH7.2, 1mM EDTA, 10mM NaCl, 0.1%SDS). 0.3mL fractions were collected, the fractions were counted and the first peak was pooled.

2.12 DNA hybridizations

DNA hybridizations were performed by the dextran sulfate method as described by Wahl et al.(1979). The hybridization of the probe to the nitrocellulose blots was performed in a Phillips Seal-a-bag. Prehybridization conditions were for 4 hours as follows:5X SSC, 250µg/mL denatured herring sperm DNA, 0.5M $NaPO_4$, pH6.5, 0.1% glycine, 0.01% BSA, 0.01% Ficoll, 0.01% PVPD, 50% formamide. Hybridization conditions were as follows: 5X SSC, 0.05M $NaPO_4$, 125 µg/mL heat denatured herring sperm DNA, 0.005% BSA, 0.05% Ficoll, 0.05% PVPD, 50% formamide. 10^7 cpm of nick translated probe was added to the reaction, and the hybridization was carried out at 43°C for 18 hours. The filters were then washed 4X at room temperature with 0.1% SDS, 2X SSC, and twice with 0.1% SDS, 0.1X SSC at 65°C. Each wash was for 15 minutes. Nitrocellulose filters were then autoradiographed at -70°C for 3 to 18 hours with a Dupont lightening plus intensifying screen. The film used for autoradiography was Kodak XA-R.

CHAPTER III

RESULTS

3.1 The isolation of H plasmid DNA

To perform molecular studies of the H plasmid group, it was necessary to first isolate plasmid DNA in a form which was relatively free of contamination from other cellular components such as chromosomal DNA, protein, or RNA. Rapid isolation techniques for large plasmids (Casse et al. 1979; Crosa & Falkow, 1981), are suitable for plasmid detection, but the purity and concentration of plasmid DNA obtained by these methods are unsuitable for restriction enzyme analysis or nicked translation since the yields of large plasmids are low. The first part of this study was therefore concerned with choosing a method which could be applied to the isolation of these plasmids by cesium chloride ethidium bromide density gradient centrifugation. Several methods have been described for the isolation of large plasmid DNA (Hansen & Olsen, 1978; Currier & Nester, 1976). These methods take advantage of the physical properties of plasmid DNA (termed covalently closed circular, or CCC) versus the physical nature of the chromosome.

Three methods were used to isolate H plasmid DNA, and these are outlined in section 2.4. Many of the observations

concerning the isolation of these plasmids were qualitative. In all cases, the plasmids were cultured in RM as it had been suggested that a predominance of superhelical plasmids was not always noted with large plasmids grown in L-broth, although it was favored in a minimal medium (Colman et al, 1978).

Initially, a scaled up version of a rapid isolation technique described by Kado & Liu (1981) was used. This method had been reported for the isolation of plasmids up to 350 Md in size. This method is based on the premise that plasmid DNA can be separated from chromosomal DNA by treatment with alkali. Plasmids have been shown to be resistant to strand separation within a narrow range of pH(12.0-12.5)(Birnboim & Doly, 1979), whereas chromosomal DNA will denature in this pH range. The lysates were then heat treated to completely denature and abolish the secondary structure of the linear DNA. The single stranded chromosomal DNA was then separated from the plasmid DNA by phenol extraction. The method was modified several times in order to isolate H plasmid DNA. When the method was scaled up with no major modification, a faint CCC band was observed in the CsCl ethidium bromide gradients. One problem of note which will be detailed later was the appearance of a third band in the CsCl gradients. That is, there was one band comprised

of chromosomal and open circular DNA, one band of plasmid DNA, and one band in between these two bands. The technique was modified by the treatment of the lysate with RNAse, followed by a protease treatment (either pronase or proteinase K). The addition of these two treatments had two effects: there was an apparent increase in the plasmid yield (as judged by an increase in the lower band), and only two bands were noted in the gradients, an upper band containing chromosomal and open circular DNA, and a lower band containing CCC DNA. It might be inferred from this that the middle band found in the gradients was a DNA-protein complex, and that the protease treatment by removing this interaction allowed the DNA in this band to now band with the other plasmid DNA. In spite of significant improvements, however, this method was tedious and the plasmid DNA was not concentrated enough for further studies. To estimate the concentration of DNA in these preparations by absorbance at 260nm was difficult because they were dilute (approximately 5 ug total). This was thought to be an insufficient yield from 500mL of culture.

An alternate method utilizing sarkosyl lysates was then tried. In this procedure, cells are lysed with sarkosyl, an ionic detergent, and the whole cell lysates are then directly loaded on cesium chloride ethidium bromide density

gradients. The separation of plasmid DNA from chromosomal DNA is thus dependent on a difference in buoyant density alone, and no prior attempt is made to eliminate chromosomal DNA. This method has also been reported as being effective in the isolation of large plasmids (Lin and Kado, 1977). The method was again scaled up and an RNAse and proteinase K step was included as suggested by Skeikholeslam et al. (1979). A shearing step which shears the chromosomal DNA, and was suggested in the original method was not included. In an initial experiment, half of the lysate was sheared by slowly pipetting the lysate up and down in a 5mL pipette. No difference was noted between lysates treated in this manner from unsheared lysates. Perhaps the handling of the lysate in the isolation or loading of the lysate onto CsCl gradients was sufficient to shear the chromosomal DNA. The use of a protease was included in this method because it had proven effective with the Kado and Liu method, and also because protease treatment was reported to be effective in freeing plasmid DNA from the folded chromosomal matrix (Kado, 1975). The usefulness of a protease with this technique is in question. Two independent experiments were done without the addition of a protease. In one experiment, CCC bands were noted and in another, no CCC bands were found. It may be that the sarkosyl alone is effective in

liberating the plasmids from the chromosomal matrix. The inclusion of a protease step did not however have any adverse effect on the isolation of these plasmids, and was included in subsequent isolations. The DNA isolated using this technique was of sufficient quantity and purity for further experiments. The yield was in the range of 10-20 ug/mL and 1.5 mL were obtained from 500 mL of cells as determined by the fluorescence method. The plasmids isolated by this technique were used in restriction enzyme digestions and Southern transfer hybridizations experiments.

To verify that the band isolated from the CsCl density gradients was plasmid DNA, the DNA preparations were examined by electron microscopy. An electron micrograph of an H plasmid is given in Figs. 2 and 3. The preparations were also run on a 0.5% agarose gel to verify that they contained CCC DNA (Fig.4).

One additional technique was used to isolate H plasmid DNA. This was a scaled up version of the alkaline lysis method of Birnboim and Doly (1979). Although this technique was not specifically reported for the isolation of large plasmids, it proved to be effective in the isolation of H plasmids, and was used in later experiments. Unlike the Kado and Liu method, the chromosomal DNA was salt precipitated, rather than heat cleared and phenol

extracted. In initial trials of this method no protease treatments were included. Again three bands were noted in the gradients (Fig.5). The two lower bands were extracted as separately as possible from the gradients. Agarose gel electrophoresis of these species (0.5% agarose) revealed that they were two DNA species of close but resolvable molecular weight. There was a slight degree of cross contamination of these two bands due to their close proximity in the CsCl gradients. These bands were then examined by electron microscopy, and both contained CCC DNA. No unusual topological forms such as nicked catenenes were noted in the middle bands. These findings may suggest that the middle band may have been a DNA-protein complex. However, in this case, protease treatment of the DNA just prior to CsCl ethidium bromide density gradient centrifugation did not result in the elimination of the middle band in the gradient. In order to further examine the possibility that this was a DNA-protein complex, the ethanol precipitated DNA was resuspended in a buffer containing sarkosyl described in section 2.4(A), and the preparation was treated with proteinase K prior to ultracentrifugation in CsCl gradients. Again three bands were found in the gradients. These conditions were more optimal for proteinase K than in TE buffer as above. The reason for the appearance of the

middle band in the gradients is still unclear, however, the DNA isolated in this manner was suitable for restriction enzyme analysis and gave the same pattern of cleavage as the DNA isolated by the sarkosyl lysate method. Similar amounts of DNA were obtained with this method as with the sarkosyl lysate method.

Fig. 2

Electron micrograph of the HI1 plasmid pRG1251. The H plasmid here is shown as supercoiled molecules. Arrows point to pBR322. Magnification is 17355X.

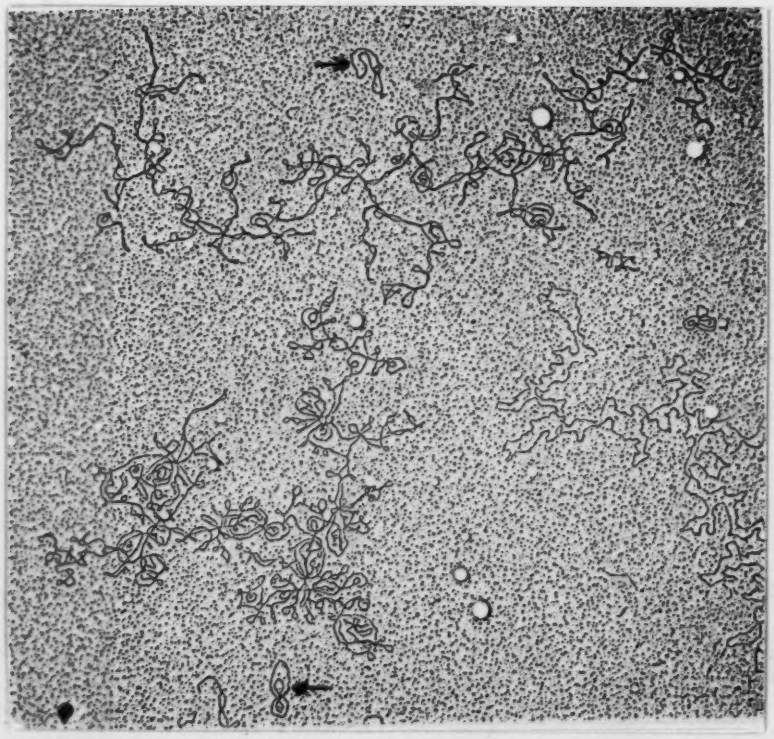


Fig. 3

Electron micrograph of a relaxed molecule of the HI1 plasmid pRG1251. Arrows point to pBR322. Magnification is 17355

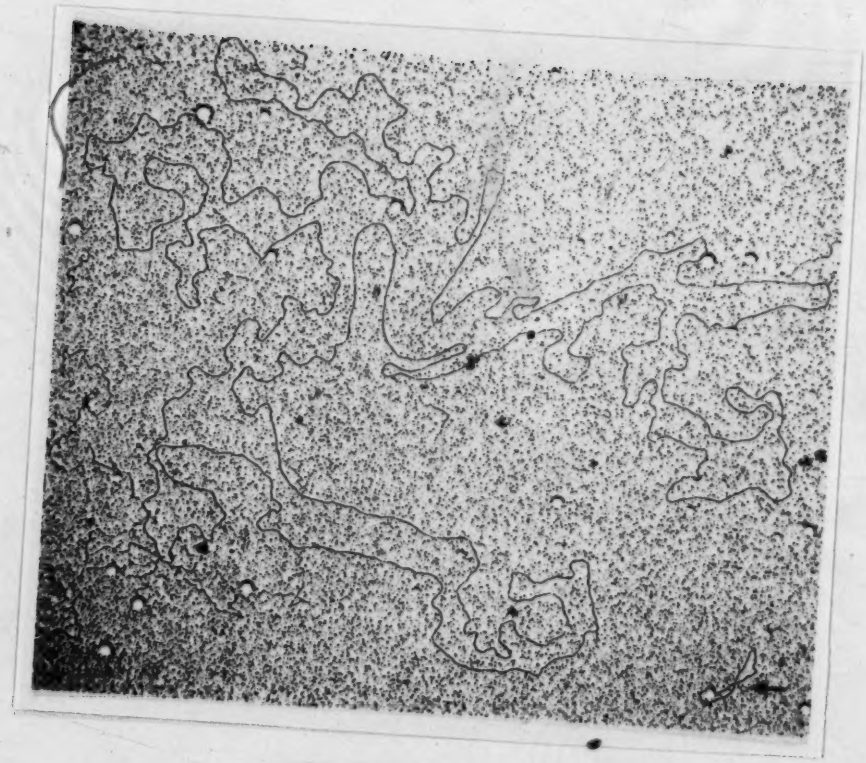


Fig. 4

Agarose gel electrophoresis of H plasmids isolated by the sarkosyl lysate method.

(A) pRG1251(HI1)

(B) R478(HI2)

(C) MIP233(HI3)

(D) bacteriophage λ DNA

samples were electrophoresed on a 0.5% agarose gel

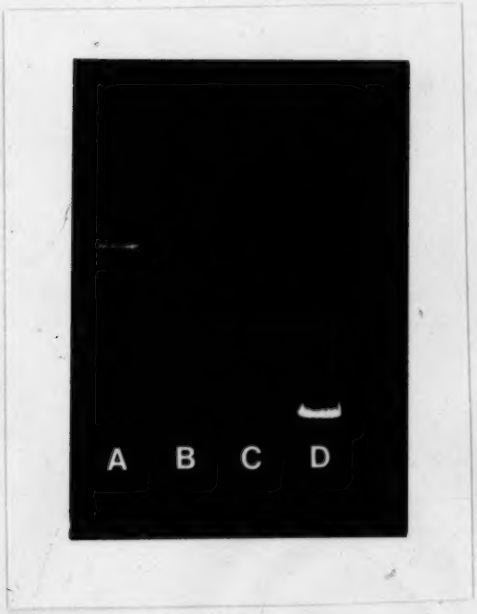

A B C D

Fig. 5

Cesium chloride ethidium bromide density gradients of H plasmids isolated by the method of Birnboim and Doly(1979), showing the appearance of three bands in the gradients.

3.2 Restriction Enzyme Analysis

Initially six restriction enzymes were chosen to digest a representative plasmid of each of the three H plasmid subgroups. These enzymes were chosen because they cut the well characterized plasmid pBR322 once. All have a six base pair recognition sequence and thus cut the DNA less frequently then those enzymes with a four base pair recognition sequence. The enzymes chosen for this survey were AvaI, BamHI, EcoRI, HindIII, PstI, and SalI. No common bands were noted amongst the plasmids of the three HI subgroups, although there were trends in the frequency of cutting with a given enzyme. For all three plasmids used, the following trends were established. AvaI, BamHI, HindIII, and EcoRI, were frequent cutters. Of these, AvaI, BamHI, and HindIII, were unsuitable for further analysis as they generated too many fragments. EcoRI gave a clearly resolved pattern generating between 27 and 39 fragments. SalI a moderate cutter, gave between 19 and 29 fragments, and PstI, was an infrequent cutter giving between 8 and 19 fragments. In each case, the smallest plasmid, pRG1251(HI) generated the least number of fragments. Digests of these plasmids with EcoRI, SalI, and PstI are given in Figs. 6, 7 and 8.

Due to the observed trends in frequency of cutting of these plasmids with a given enzyme, a further survey of enzymes was done with the HI1 plasmid, pRG1251, to look for other enzymes which would give a clearly resolved restriction enzyme pattern. These enzymes were XbaI, PvuII, AvaII, AccI, Sau96I, KpnI, HpaI, and BglII. All of these enzymes have a six base pair recognition sequence except for Sau96I, which has a 5 base pair recognition sequence. Four of these enzymes, HpaI, KpnI, and XbaI, and BglII, do not cut pBR322. PvuII cuts pBR322 once, and AccI cuts pBR322 twice. The other enzymes cut pBR322 more frequently, but were available in the restriction enzyme collection in the laboratory. Fig. 9 shows pRG1251 digested with these enzymes. XbaI, is an infrequent cutter. PvuII, AvaII and Sau96I were frequent cutters, but unsuitable because the largest fragments generated with these enzymes were in the 9-4kb range, indicating that most of the DNA from these digests was in the lower molecular weight ranges and are not suitable for resolution in an agarose gel system. BglII and HpaI were frequent cutters like EcoRI. KpnI was a moderate cutter, like SalI. The three sub-group plasmids were then digested with BglII, XbaI, and HpaI. These digests are shown in Figures 10, 11 and 21.

For MIP233(HI3), the digestion products with XbaI, were all large and unresolved in the tris-borate system used here. BglII gave a frequent pattern of cleavage, and HpaI was a frequent cutter for all three subgroup plasmids, however it generated too many fragments from the HI2, and HI3 plasmid for meaningful analysis.

Double digests using a combination of infrequent and moderate cutters were done to more clearly resolve the fragments of high molecular weight. The three enzymes used were PstI, XbaI, and SalI. XbaI and SalI are usually digested in high salt buffer. PstI digestions are usually done in medium salt buffer. A PstI digest of pRG1251 in high salt buffer was first done to see if this enzyme would function in high salt. PstI was able to digest this plasmid to completion in high salt buffer and therefore double digestion were performed in high salt buffer. Double digestion of PstI/XbaI, and PstI/SalI are shown in Figures 12 and 13. Double digestions were of some use in resolving the bands of high molecular weight, notably pRG1251, with PstI/SalI, and MIP233 with PstI/XbaI. Using double digests, more bands were noted closer to the largest band of lambda digested with HindIII (23.7kb). These bands could be more accurately sized. The incHII plasmid digested with EcoRI, did not appear to have any common bands with the HI subgroup plasmids used in this study. (Fig.18)

Table 13 lists the total molecular weight of the fragments obtained with various enzymes. The published molecular weights as previously determined by agarose gel electrophoresis is also given in this table. The values obtained from straight addition of the molecular weights of the fragments from the digests are less than the published values. This may be because some of the bands have a multiplicity > 1. Upon inspection of the various gels, some of the bands appear to have a multiplicity greater than 1. Exact determination of the multiplicity of these bands would have to be made by prolonged electrophoresis of the digests to resolve these fragments or by the use of densitometry. With the frequent cutter, EcoRI, identification of these bands is more obvious then with the moderate cutter SalI, or the infrequent cutter PstI, since the increase in intensity of the large molecular weight fragments obtained with PstI or SalI may be a result of increased fluorescence due to the binding of a greater amount of ethidium bromide. Unequivocal determination of the multiplicity of the bands in a given digest would have to be made for purposes of restriction mapping and would be necessary to determine the total molecular weight of these plasmids.

Fig. 6

Agarose gel electrophoresis of H plasmids digested with the frequent cutter EcoRI .

(A) phage λ digested with HindIII

(B) pRG1251(HI1)

(C) R478(HI2)

(D) MIP233(HI3)

Samples were run on a 0.8% agarose gel as described in the methods and materials

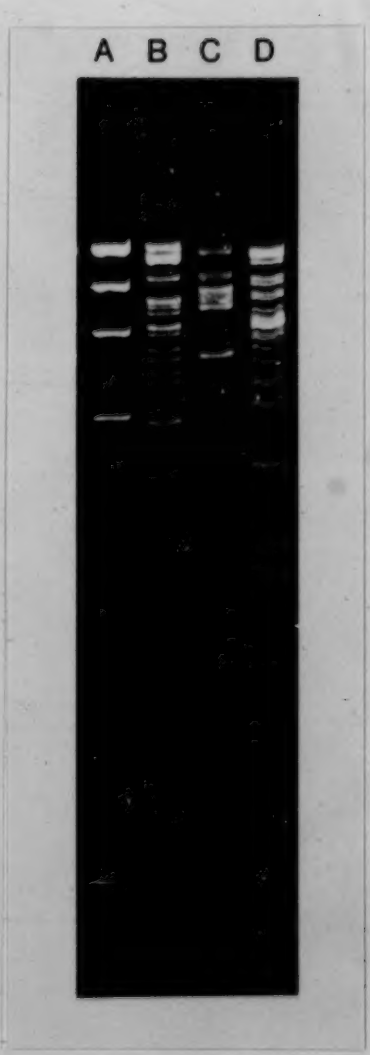

A B C D

Table 7

Sizes of fragments generated by EcoRI

Fragment #	pRG1251	R478	MIP233
1.	29.91	23.79	22.64
2.	19.77	13.95	17.56
3.	13.56	11.88	13.18
4.	9.94	10.36	12.18
5.	9.19	9.55	10.58
6.	8.56	8.56	9.94
7.	7.52	8.00	8.71
8.	7.00	7.64	8.00
9.	6.23	6.81	7.76
10.	5.81	6.64	7.52
11.	5.34	6.16	7.00
12.	5.03	5.50	6.72
13.	4.14	5.28	6.31
14.	3.94	5.23	5.81
15.	3.63	4.60	5.13
16.	3.33	3.85	4.48
17.	2.83	3.54	4.08
18.	2.70	3.35	3.91
19.	2.17	3.28	3.78
20.	2.09	3.17	3.65
21.	2.05	3.04	3.50
22.	1.96	2.84	3.35
23.	1.91	2.75	3.06
24.	1.89	2.59	2.99
25.	1.76	2.54	2.72
26.	1.70	2.42	2.67
27.	1.67	2.37	2.57
28.		2.24	2.51
29.		2.14	2.28
30.		2.07	2.23
31.		2.02	2.09
32.		1.97	2.06
33.		1.83	2.01
34.		1.79	1.92
35.		1.74	1.80
36.			1.78
37.			1.76
38.			1.69
39.			1.65

Fig. 7

Agarose gel electrophoresis of H plasmids digested with the moderate cutter SalI .

(A) phage λ digested with HindIII

(B) pRG1251(HI1)

(C) R478(HI2)

(D) MIP233(HI3)

Samples were run on a 0.5% agarose gel as described in the methods and materials.

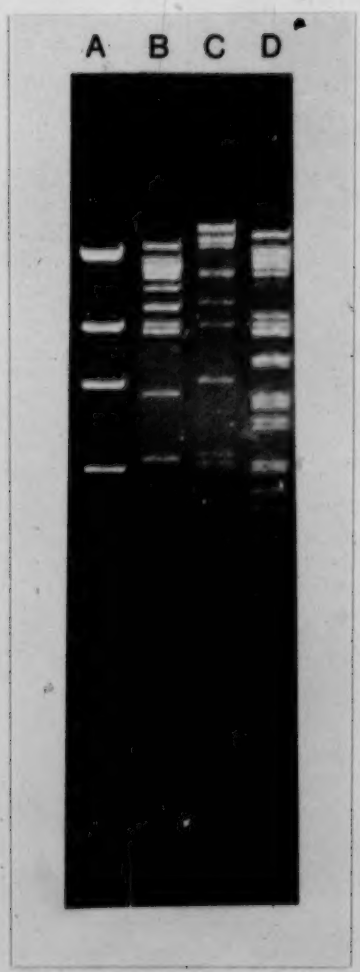

A
B
C
D

Table 8

Sizes of fragments generated by SalI

Fragment #	pRG1251	R478	MIP233
1.	28.09	47.27	39.94
2.	22.17	34.53	28.09
3.	19.74	21.51	25.22
4.	17.76	13.83	22.86
5.	15.40	11.23	20.88
6.	12.53	7.40	19.20
7.	10.85	4.81	12.07
8.	10.16	4.62	11.23
9.	6.53	3.25	10.49
10.	4.62	2.19	8.59
11.	3.28	1.81	8.23
12.	3.04		6.61
13.	2.87		6.45
14.	2.59		6.23
15.	2.03		5.82
16.	1.91		5.57
17.	1.56		4.62
18.	1.45		4.40
19.	1.32		4.08
20.			3.76
21.			3.28
22.			2.78
23.			2.59
24.			2.51
25.			2.36
26.			1.94
27.			1.71
28.			1.49
29.			1.35

Fig. 8

Agarose gel electrophoresis of H plasmids digested with the infrequent cutter PstI

(A) phage λ digested with HindIII

(B) pRG1251(HI1)

(C) R478(HI2)

(D) MIP233(HI3)

Samples were run on a 0.5% agarose gel as described in the methods and materials.

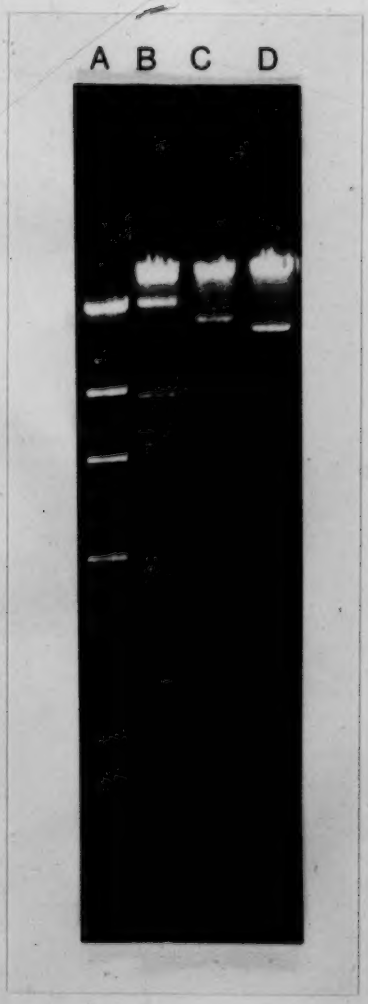

A B C D

Table 9

Sizes of Fragments generated by Pst I

Fragment #	pRG1251	R478	MIP233
1.	47.27	53.78	47.27
2.	37.66	47.27	17.76
3.	23.60	20.30	6.38
4.	9.84	4.72	5.95
5.	7.69	2.85	4.96
6.	2.85	1.97	4.81
7.	1.98	1.70	4.62
8.	1.46	1.51	4.53
9.			4.01
10.			3.83
11.			3.33
12.			3.25
13.			2.71
14.			2.59
15.			2.03
16.			1.91
17.			1.53
18.			1.42
19.			1.27

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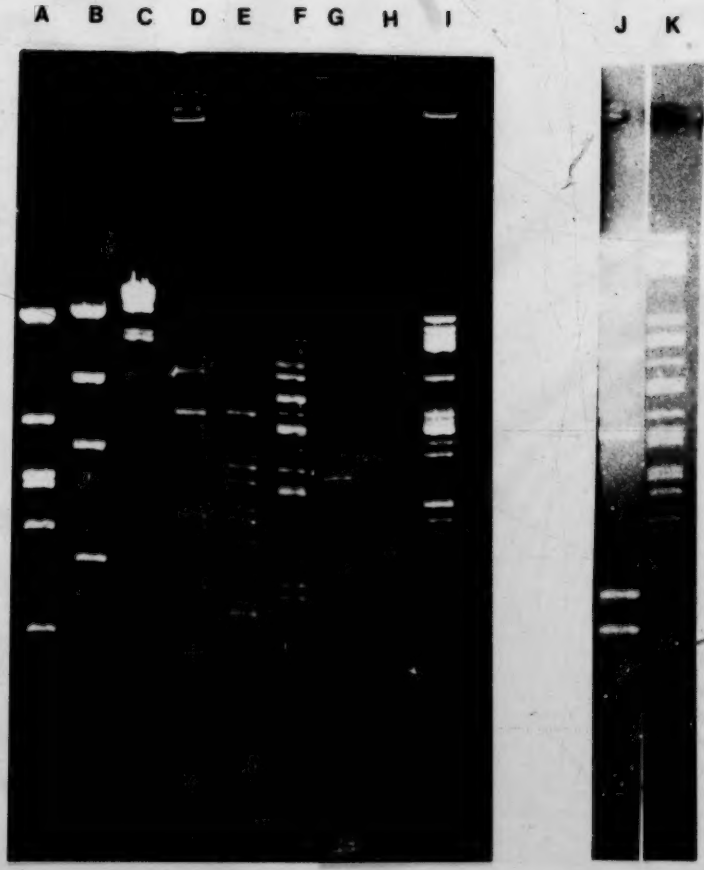

A B C D E F G H I
J K

Fig. 9

Agarose gel electrophoresis of the HI1 plasmid pRG1251 digested with various restriction enzymes

(A) phage λ digested with EcoRI

(B) phage λ digested with HindIII

(C) pRG1251 digested with XbaI

(D) pRG1251 digested with PvuII

(E) pRG1251 digested with AvaII

(F) pRG1251 digested with AccI

(G) pRG1251 digested with Sau96I

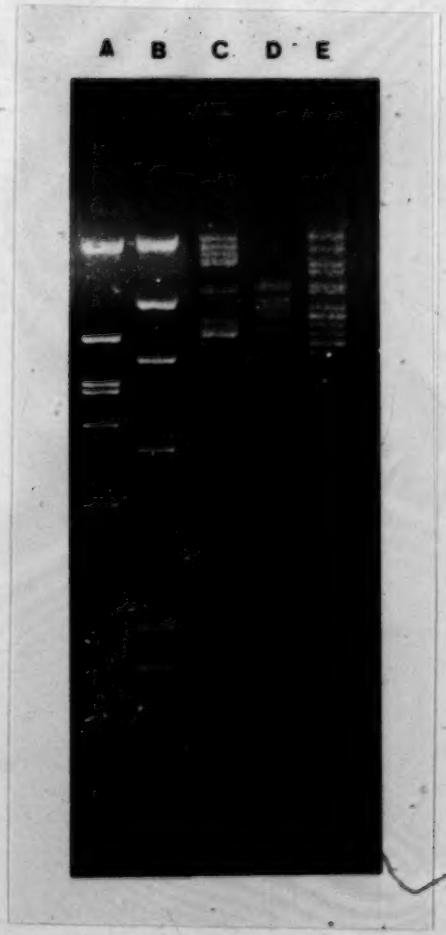
(H) pRG1251 digested with KpnI

(I) pRG1251 digested with HpaI

(J) phage λ digested with HindIII

(K) pRG1251 digested with BglII

Samples were run on a 0.5% agarose gel as described in the methods and materials.


A
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E

Fig. 10

Agarose gel electrophoresis of H plasmids digested with HpaI

(A) phage λ digested with EcoRI

(B) phage λ digested with HindIII

(C) pRG1251(HI1)

(D) R478(HI2)

(E) MIP233(HI3)

Samples were run on a 0.8% agarose gel as described in the methods and materials

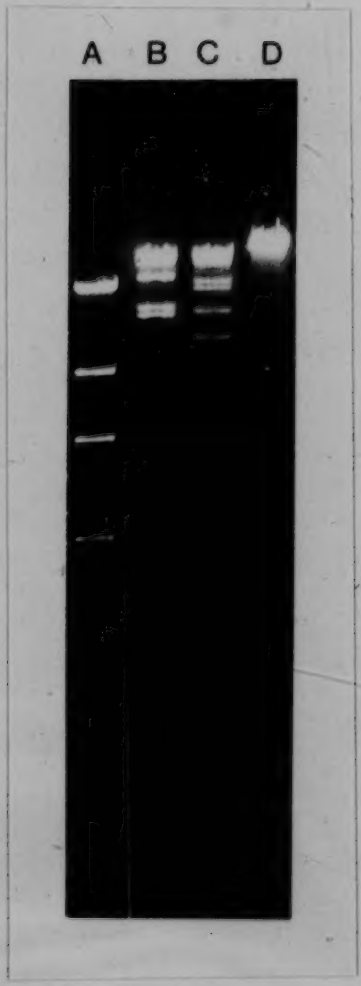

A
B
C
D

Table 10

Sizes of fragments generated by Xba I

Fragment #	pRG1251	R478	MIP233
1.	53.30	57.95	108.36
2.	41.21	43.31	
3.	29.58	28.56	
4.	19.12	25.07	
5.	17.43	18.67	
6.	13.55	14.22	
7.	5.93	5.87	
8.	4.12		

Fig. 12

Agarose gel electrophoresis of H plasmids digested with PstI and XbaI

(A) phage λ digested with HindIII

(B) pRG1251(HI1)

(C) R478(HI2)

(D) MIP233(HI3)

samples were run on a 0.5% agarose gel as described in the methods and materials.

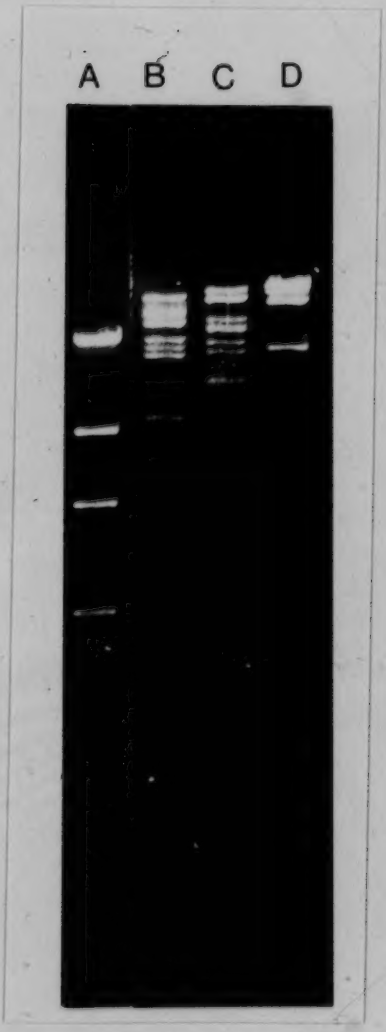

A
B
C
D

Table 11

Sizes of fragments generated by Pst I and Xba I

Fragment#	pRG1251	R478	MIP233
1.	48.20	66.91	66.90
2.	33.11	48.20	57.95
3.	28.56	31.85	43.31
4.	22.32	27.61	6.84
5.	20.60	22.95	6.36
6.	18.67	20.08	5.25
7.	11.04	15.06	5.12
8.	8.51	6.05	4.81
9.	6.36	5.03	4.54
10.	6.11	3.02	4.22
11.	4.26	2.12	4.03
12.	3.00	1.94	3.57
13.	2.14	1.88	3.45
14.	1.60		2.86
15.			2.72
16.			1.93
17.			1.79
18.			1.66

Fig. 13

Agarose gel electrophoresis of H plasmids digested wtih PstI and SalI.

(A) phage λ digested with HindIII

(B) pRG1251(HI1)

(C) R478(HI2)

(D) MIP233(HI3)

samples were run on a 0.5% agarose gel as described in the methods and materials.

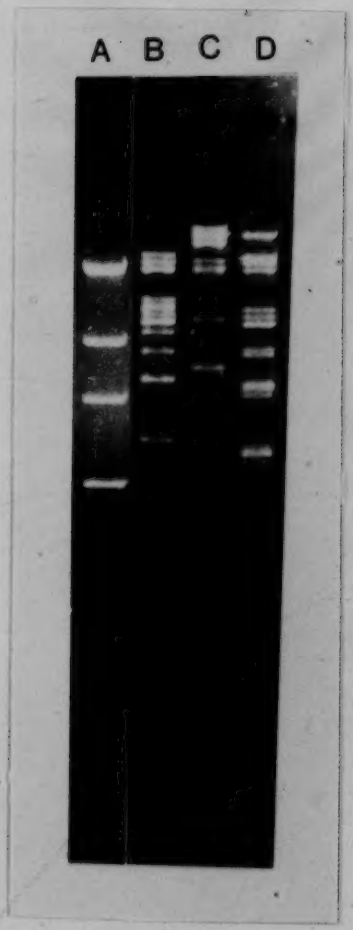

A B C D

Table 12

Sizes of fragments generated by digestion with PstI and SalI

fragment #	pRG1251	R478	MIP233
1.	24.39	50.32	39.94
2.	22.17	42.13	24.39
3.	19.20	37.97	22.17
4.	13.55	33.03	12.53
5.	12.53	20.30	12.30
6.	12.30	11.23	11.43
7.	11.43	4.76	10.67
8.	10.67	2.78	8.59
9.	9.84	2.49	8.35
10.	8.47	2.06	6.77
11.	4.81	1.87	6.38
12.	4.62	1.70	4.67
13.	3.36	1.65	4.45
14.	3.14	1.23	4.32
15.	2.99	5.11	4.12
16.	2.65	1.32	3.73
17.	1.75		3.48
18.	1.69		3.36
19.	1.56		2.78
20.	1.49		2.61
21.	1.32		2.36
22.			2.22
23.			2.06
24.			1.97
25.			1.85
26.			1.70
27.			1.57
28.			
29.			
30.			

Table 13

Summary of the total molecular weights of the fragments generated by various restriction enzymes

	Plasmid		
	pRG1251	R478	MIP233
Enzyme			
EcoRI	165kb	186kb	213kb
SalI	167	152	251
PstI	132	134	218
XbaI	165	193	(?)
PstI/XbaI	214	249	227
PstI/SalI	173	216	210
Given M.W.[a]	184	254	230

a pRG1251:120Md(183kb),Taylor & Levine,1980

R478:166Md(256kb), *in 'DNA Insertion Elements, Plasmids and Episomes.'* A.I. Bukari, J.A. Shapiro, S.L. Adhya, eds. Cold Spring Harbor Laboratory, New York.p.133.

MIP233:150Md(230kb),Bradley et al, 1982.

3.3 Estimation of the fragment sizes generated by restriction enzymes

The sizes of fragments generated by various restriction enzymes were predicted using a computer program. Accurate estimation of the fragments sizes with this program is limited within the size ranges of the molecular weight standards (see Appendix 1) . Analysis of the effectiveness of this program in the upper molecular weight ranges is given by the following results. Table 14 lists the sizes of fragments generated in this range for pRG1251 digested with PstI from a number of gels. The program was consistent in predicting the sizes of these fragments. Furthermore, these did agree with the values obtained from electron microscopy of this digest(see section 3.4). In one digest of this subgroup plasmid with PstI, a linear lambda standard was included which is an appropriate molecular weight standard in the size range of the large molecular fragments of the H plasmids digested with PstI. Inclusion of this standard had little effect on predicting the sizes of these fragments(see Table 15).

The sizes of the fragments in the upper molecular weight range are listed to two decimal places as they appeared on the computer printout. These sizes should however be regarded as accurate only to the degree indicated by their standard deviation (Table 14). For fragments whose molecular weight is within the molecular weight range covered by the standard curve, the error is smaller, and in some cases listing of the values to two decimal places was necessary to differentiate between fragments .

Table 14

Sizes of the large fragments of pRG1251 digested with PstI from several gels.

Gel #	Sizes
1.	a) 43.72 b) 33.75
2.	a) 45.63 b) 37.55
3.	a) 47.26 b) 37.97
4.	a) 41.47 b) 33.63

Standard deviation of fragment a=2.5Kb mean=44.52
Standard deviation of fragment b=2.3Kb mean=35.72

Sizes of fragments are given in Kilobase pairs.

Table 15

Sizes of large fragments from PstI digestion as predicted with and without a λlinear marker included in the standard curve.

	pRG1251	R478	MIP233
λlinear not included	41.46 33.63	57.88 46.82	50.03
λlinear included	41.18 34.37	57.23 46.43	49.57

Sizes of fragments are given in kilobase pairs.

3.4. Analysis of digestion with PstI

As was mentioned earlier in section 3.2 PstI is an infrequent cutter. For each plasmid, one or two intense bands were noted in the upper molecular weight range (40-50kb). Since size resolution in this range of molecular weights is difficult, the question was raised as to whether these bands were a composite of unresolved DNA fragments of very high molecular weight which migrate to the same region of the gel, or whether these represent a single DNA species. This was of particular note when the digests were subjected to electrophorsis on a 0.8% agarose gel. As well, it must be determined whether digestion with this enzyme was complete, and whether these bands were products from incomplete digestion.

In order to answer these questions, a digest of pRG1251 with PstI was examined by electron microscopy for very large fragments of DNA which may be present in these bands. Random fields of DNA were photographed and the molecules were measured with a digitizer. The sizes of these fragments were determined versus pBR322 (4,635kb), which was included in the DNA preparations for electron microscopy. There was no evidence of very large DNA molecules in this preparation. A profile of the fragment sizes obtained from this analysis is given in Fig.14.

The number of molecules examined were not sufficient to accurately size these fragments. It was clear, however, that the bands in the upper molecular weight range of the PstI digest did not comprise very large fragments of DNA which were unresolved.

To determine if digestion with PstI was complete, bacteriophage lambda DNA was added to the digests of these plasmids, and the products from this digestion were subject to electrophoresis on a 0.5% agarose gel (Fig.15). The digestion with PstI was considered to be complete based on this result.

Fig. 14

Histogram of molecular weights of PstI digest of pRG1251.

The fragment sizes were determined versus a pBR322 molecular weight standard.

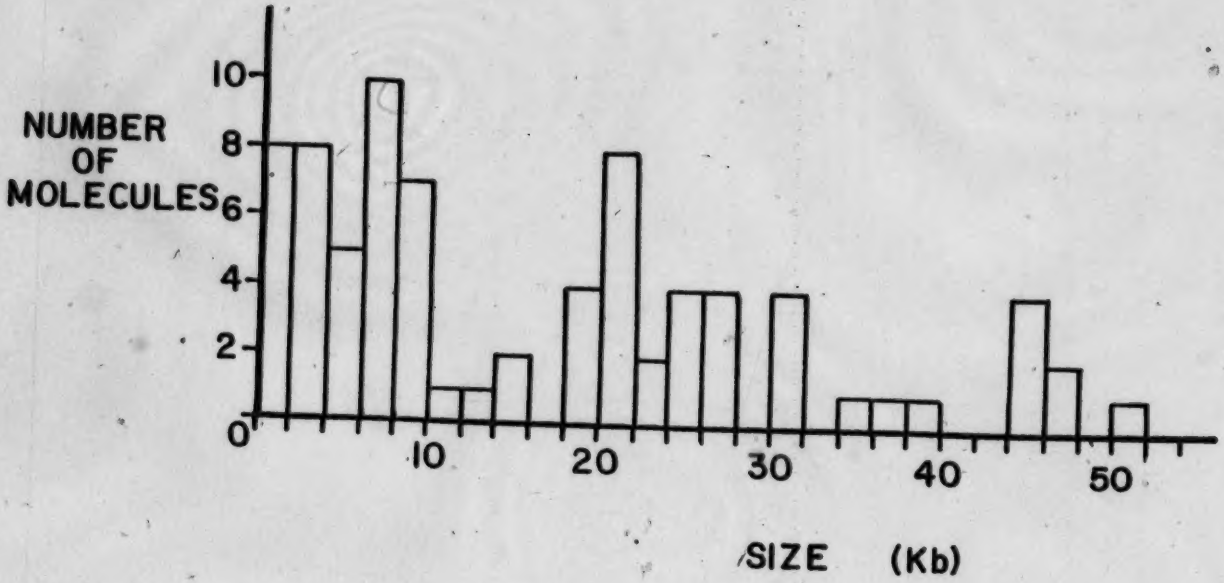

NUMBER OF MOLECULES
10
8
6
4
2
0
10
20
30
40
50
SIZE (Kb)

Fig. 15

Agarose gel electrophoresis of H plasmids digested with PstI, and bacteriophage λ internal controls.

(A) phage λ digested with EcoRI

(B) phage λ digested with HindIII

(C) pRG1251(HI1) digested with PstI

(D) R478(HI2) digested with PstI

(E) MIP233(HI3) digested with PstI

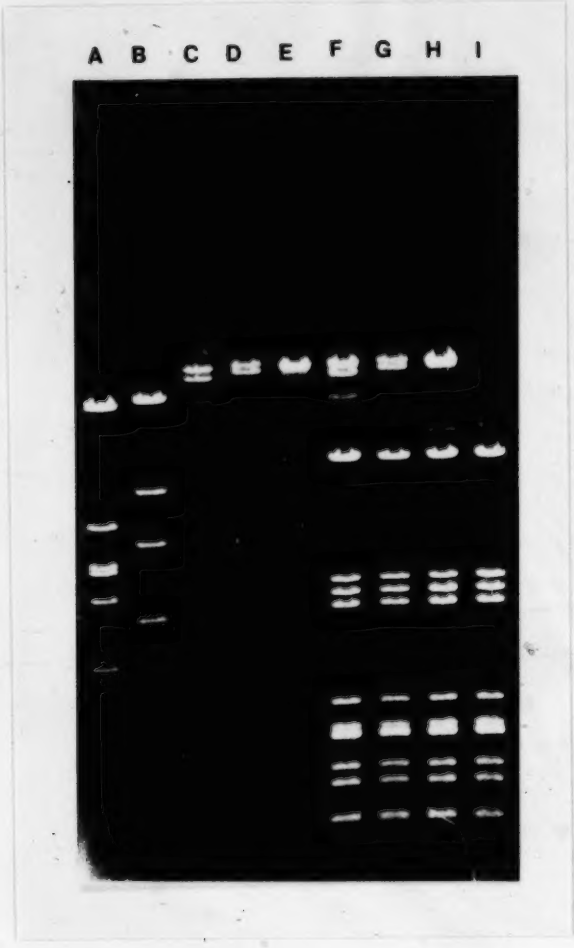
(F) pRG1251 and phage λ DNA digested with PstI

(G) R478 and phage λ DNA digested with PstI

(H) MIP233 and phage λ DNA digested with PstI

(I) phage λ DNA digested with PstI

Samples were run on a 0.5% agarose gel as described in the methods and materials.


A B C D E F G H I

3.5. Restriction enzyme cleavage within a subgroup

The digestion patterns of plasmids within a given HI subgroup were investigated. A group of five HI1, and five HI2 plasmids were compared from various geographical locations and different host bacteria (see Table 4). These plasmids were isolated by the method of Birnboim and Doly as modified in Section 2.4(D). The HI1 plasmids were digested with EcoR1, and their cleavage patterns examined. The plasmids within the HI1 subgroup had similar restriction enzyme cleavage patterns and there were many bands common to all the plasmids examined. Similarly, when the HI2 plasmids were digested with EcoRI, they also showed extensive similarities in their cleavage patterns. However, there were fewer common bands shared by this group than by the HI1 plasmids. These HI2 plasmids were isolated from a variety of genera as opposed to the HI1 plasmids which were all originally obtained from strains of Salmonella(see Table 2). This may account for the HI2 plasmids showing fewer similarities than the HI1 plasmids in their restriction enzyme cleavage patterns.

Fig. 16

Agarose gel electrophoresis of HI1 plasmids digested with EcoRI

(A) phage λ digested with EcoRI

(B) phage λ digested with HindIII

(C) pRG1251

(D) pRG1271

(E) pRG1284

(E) R27

(F) TP124

samples were run on a 0.7% agarose gel as described in the methods and materials.

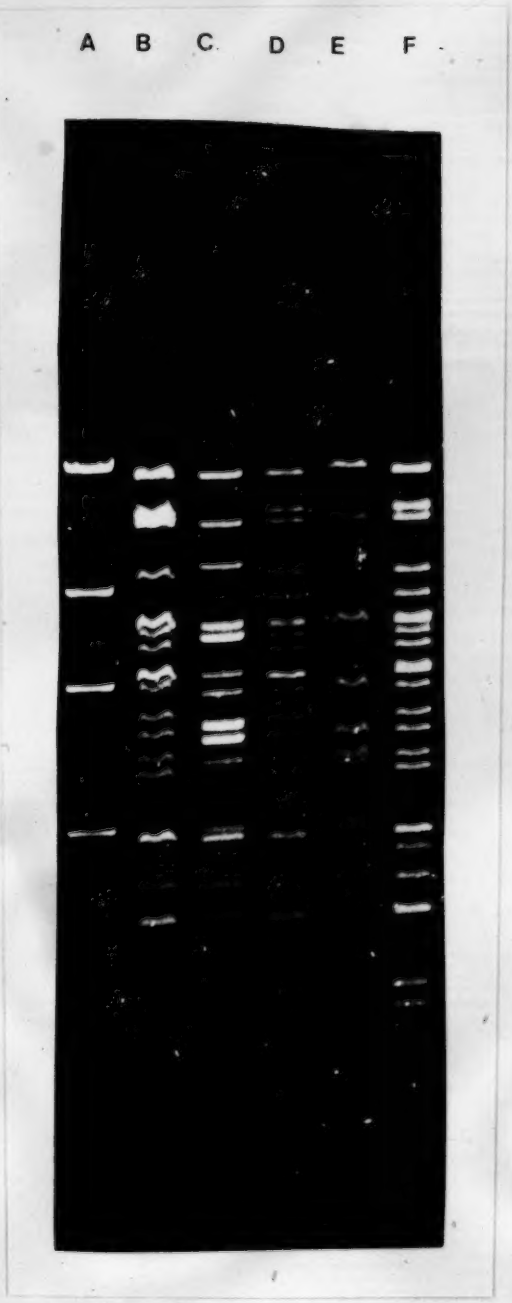

A
B
C
D
E
F

Fig. 16

Agarose gel electrophoresis of HI1 plasmids digested with EcoRI

(A) phage λ digested with EcoRI

(B) phage λ digested with HindIII

(C) pRG1251

(D) pRG1271

(E) pRG1284

(E) R27

(F) TP124

samples were run on a 0.7% agarose gel as described in the methods and materials.

A B C D E F

3.5 DNA Homology Studies

The following restriction enzyme digests of the three HI subgroup plasmids were transferred to nitrocellulose: EcoRI, PstI, SalI, XbaI, BglII, PstI/XbaI, and PstI/SalI. The fragments from these digests were probed for homology with a nick-translated HI1 plasmid, pRG1251. The results from these experiments show that there was a small amount of DNA homology between the HI subgroup plasmids. The use of several enzymes afforded an analysis of the distribution of fragments showing DNA homology in various molecular weight ranges. With most enzymes, either singly or in combination, some DNA homology was noted in the fragments of higher molecular weight. This was true for all classes of enzymes (frequent, moderate and infrequent). Double digestion with a combination of enzymes did not reduce the number of fragments in the upper molecular weight range except in the case of MIP233 digested with XbaI, in which the large fragments were unresolvable. Double digestion with PstI and XbaI or SalI did not give further information as the fragments showing homology were identical to those of digestion with PstI alone.

One very important exception to the observation that DNA homology was found mainly in the upper molecular weight

range was with the restriction digest of MIP233 with PstI. The homology with this plasmid was restricted to a 2-3kb size range (see Table 18). Southern transfers of the following digests are shown in Figs. 18, 19, 20, 21: EcoRI, PstI, SalI, BglII. Double digestions are not shown as they did not reveal any further information. The sizes of the fragments showing some DNA homology with the nick-translated HI plasmid, pRG1251, are given in Tables 16, 17, 18 and 19.

In another experiment the radioactively labelled HI1 plasmid, pRG1251, was used as a probe against four other HI1 plasmids. The results from this experiment were that there was extensive DNA sequence homology within this subgroup. This is shown in Fig.22.

A small amount of homology was noted with the incHII plasmid probed with the HI1 plasmid. The fragments showing homology were in the upper molecular weight range (Fig.18).

Fig. 18

Hybridizations between H plasmids. The ^{32}P -labelled probe is the HI1 plasmid pRG1251,hybridized against EcoRI digests of the plasmids .

(A) phage λ digested with EcoRI

(B) phage λ digested with HindIII

(C) pRG1251

(D) R478(HI2)

(E) MIP233 (HI3)

(F) pHH1508a(HII)

(G) pRG1251

(H) R478

(I) MIP233

(J) pHH1508a

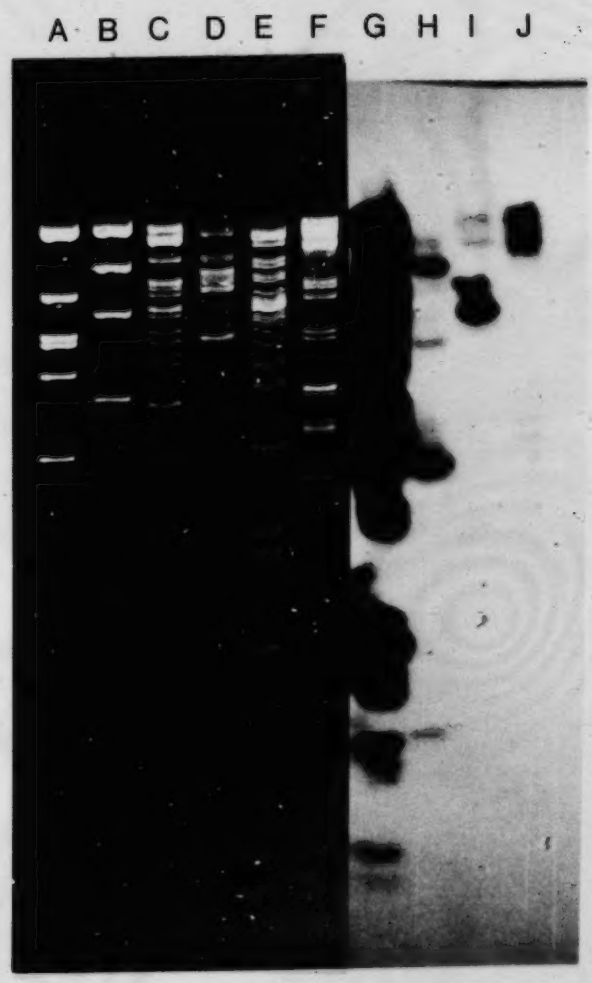

A B C D E F G H I J

Table 16

EcoRI fragments showing homology with the ^{32}P labelled plasmid pRG1251.[a]

R478(HI2)		MIP233(HI3)		pHH1508a	
fragment#	size	fragment#	size	fragment#	size
1	23.79	1	22.64	1	37.11
2	13.95	3	13.18	2	26.50
5	9.55	(5,6)	10.58	3	18.24
(6,7)	8.56		9.94	11	3.88
	8.00	(8,9)	8.00	13	3.70
(10,11)	6.64		7.76		
	6.16	20	3.65		
(17,18)	3.54				
	3.35				
33.	1.83				

fragment sizes are given in kilobase pairs

[a] Identification of the exact band showing homology was not always possible because of the close proximity of the bands in the agarose gel. When this was the case, the bands are listed in brackets. The interpretation should therefore be that any or all of the fragments given in brackets may have been responsible for the homology noted.

Fig. 19

Hybridizations between H plasmid. The ^{32}P labelled probe is the HI1 plasmid pRG1251, hybridized against SalI digests of the plasmids.

(A) phage λ digested with HindIII

(B) pRG1251

(C) R478(HI2)

(D) MIP233(HI3)

(E) pRG1251

(F) R478

(G) MIP233

A B C D E F G

Table 17

SalI fragments showing homology with the ^{32}P - labelled HI1 plasmid pRG1251 [a]

R478		MIP233	
fragment#	size	fragment#	size
(1,2)	47.27	(2,3,4)	28.09
	34.53		25.22
8	4.62		22.86
		23	2.59

sizes of fragments is given in kilobase pairs

[a] Identification of the exact band showing homology was not always possible because of the close proximity of the bands in the agarose gel. When this was the case, the bands are listed in brackets. The interpretation should therefore be that any or all of the fragments given in brackets may have been responsible for the homology noted.

Fig. 20

Hybridizations between H plasmids. The ^{32}P-labelled probe is the HI1 plasmid pRG1251, hybridized against PstI digests of the plasmids.

(A) phage λ digested with EcoRI

(B) pRG1251

(C) R478 (HI2)

(D) MIP233 (HI3)

(E) pRG1251

(F) R478

(G) MIP233

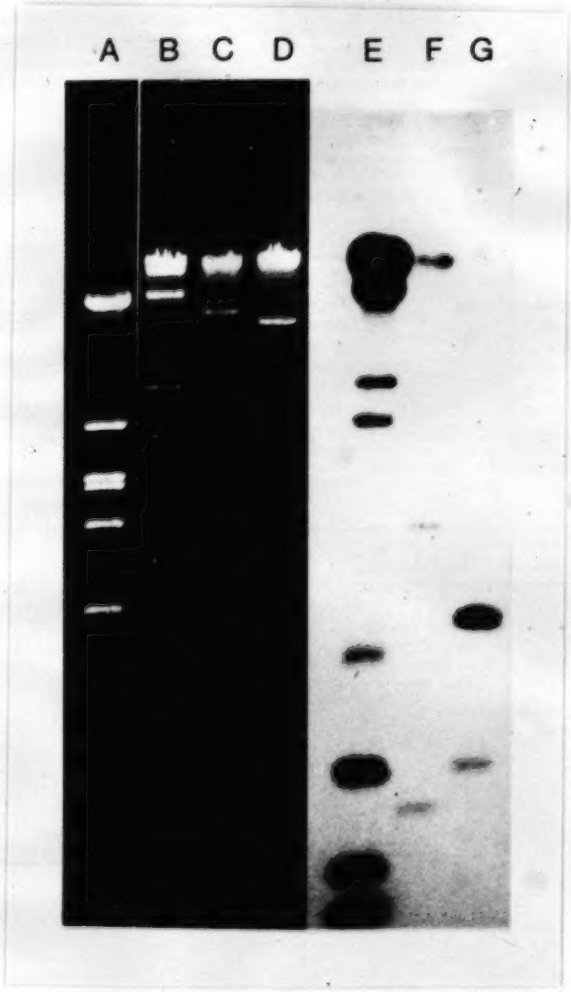

A B C D E F G

Table 18

PstI fragments showing homology with the ^{32}P labelled HI1 plasmid pRG1251.[a]

R478(HI2)		MIP233(HI3)	
fragment#	size	fragment#	size
(1,2)	51.08	(11,12)	3.33
	45.63		3.24
4	4.69	15	2.07
6	2.41		

sizes of fragments are given in kilobase pairs

[a] Identification of the exact band showing homology was not always possible because of the close proximity of the bands in the agarose gel. When this was the case, the bands are listed in brackets. The interpretation should therefore be that any or all of the fragments given in brackets may have been responsible for the homology noted.

Fig. 21

Hybridizations between H plasmids. The ^{32}P-labelled probe is the HI1 plasmid pRG1251, hybridized against BglII digests of the plasmids.

(A) phage λ digested with HindIII

(B) pRG1251

(C) R478 (HI2)

(D) MIP233 (HI3)

(E) pRG1251

(F) R478

(G) MIP233

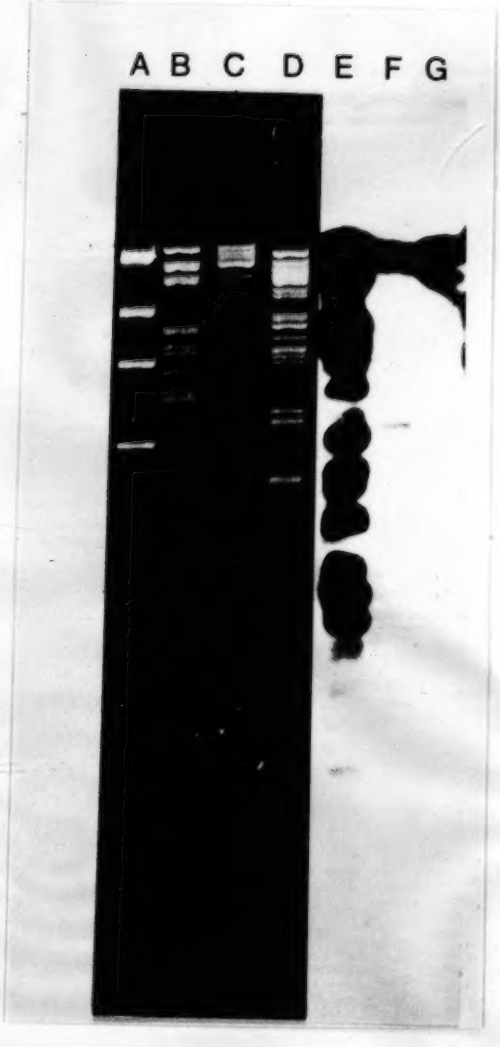

A B C D E F G

Table 19

BglII fragments showing homology with the ^{32}P-labelled HI1 plasmid pRG1251.[a]

R478		MIP233	
fragment#	size	fragment#	size
(1,2,3,4)	26.95	(1,2,3)	25.60
	23.27		20.50
	21.31		18.91
	18.01		
14	5.84		

fragment sizes are given in kilobase pairs

[a] Identification of the exact band showing homology was not always possible because of the close proximity of the bands in the agarose gel. When this was the case, the bands are listed in brackets. The interpretation should therefore be that any or all of the fragments given in brackets may have been responsible for the homology noted.

Fig. 22

Hybridizations between HI1 plasmids. The ^{32}P-labelled probe is the HI1 plasmid pRG1251, hybridized against EcoRI digests of the plasmids.

(A) R27

(B) TP124

(C) pRG1271

(D) pRG1251

(E) pRG1284

A B C D E

CHAPTER IV

DISCUSSION AND CONCLUSIONS

4.1 The Isolation of H plasmids

Since plasmid DNA represents only a small fraction of the DNA present in the cell, isolation procedures have been designed to separate this DNA from the chromosomal DNA. All these techniques take advantage of the differences in the physical properties of plasmids versus those of the chromosomal DNA. The chromosome is large, and is extracted from cells as broken linear molecules which can be removed by precipitation with tangled cell debris, whereas plasmids are generally extracted as covalently closed circles. In addition plasmids are resistant to certain chemical or physical treatments. Because plasmids exist as covalently closed circles, the DNA strands of these molecules cannot be separated without breaking one of these strands. Within a narrow range of pH(12.0-12.5) plasmids are resistant to strand separation whereas the chromosomal DNA will denature into single stranded DNA. If the pH is then adjusted to neutrality, the plasmid DNA will return to its native state, while the

chromosomal DNA will form an aggregate which can then be separated by centrifugation. Treatment of lysates with heat has a similar effect. Chromosomal DNA and plasmid DNA can be separated from each other in CsCl ethidium bromide density gradients. This separation requires that the plasmid DNA is supercoiled, that is there are no nicks in either of the strands of the plasmid DNA molecule (a nick is a breakage of a phosphodiester bond). If plasmid DNA is nicked, then the supercoiling of these molecules is removed and the plasmid DNA is termed relaxed. The separation of plasmid DNA from chromosomal DNA in these gradients is based on the fact the CCC DNA binds less ethidium bromide than linear or nicked plasmid DNA. Ethidium bromide is a dye which intercalates between the bases in DNA, causing it to unwind. As a result the chromosomal DNA is less dense than CCC DNA, and it is this difference in density which allows these two species to band at different densities in the gradient. Plasmid DNA consequently forms the lower band in the gradient (Radloff et al., 1967). From these observations of the different properties of plasmid DNA versus chromosomal DNA, it is apparent that the larger the plasmid, the more closely its properties will resemble those of the chromosome, and it therefore becomes increasingly more difficult to isolate

large plasmids like those of the H group. Large plasmids are more readily susceptible to breakage by shear force and differential removal of chromosomal DNA from large plasmid DNA by centrifugation or salt precipitation may also pose problems.

Several methods have been reported for isolating large plasmids DNA (Hansen & Olsen, 1978, Currier & Nester, 1976). The procedure described by Currier & Nester involved shearing of the chromosomal DNA, alkaline denaturation, neutralization and removal of the denatured DNA by phenol extraction, and finally recovery of the plasmid DNA by ethanol precipitation. The plasmid DNA was then purified by CsCl ethidium bromide density gradient centrifugation. Currier & Nester reported that approximately 80 - 90% of the CCC DNA was lost in the initial shearing step, but that by using large volumes of lysates, one could recover plasmid DNA from CsCl gradients. The Hansen & Olsen procedure was similar to the procedure of Currier & Nester except that the chromosomal DNA and membrane pieces are removed by salt precipitation. Currier & Nester reported that recovery of large plasmid DNA by salt precipitation gave consistently low recovery and degree of purification for large plasmids. Other methods, for example like the sarkosyl lysate method (Bazaral & Helinski, 1968) separate the large plasmid DNA from the chromosomal DNA

by buoyant density alone. In the Kado & Liu method (section 2.4), the chromosomal DNA is denatured by heat and alkali, and removed by phenol extraction.

Three methods were used in this study to isolate H plasmid DNA. Two of these methods had been specifically reported for the isolation of large plasmids. These were the method of Kado & Liu (1981) and the sarkosyl lysate method of Bazaral and Helinski (1968). H plasmid DNA was isolated by both methods, however the yields obtained from the Kado and Liu method were unsatisfactory. A third method, that of Birnboim and Doly (1979) which had not specifically been reported for the isolation of large plasmids also proved to be effective in their isolation. This method involves an alkaline lysis step followed by neutralization and removal of the aggregated chromosomal DNA by centrifugation. The membrane pieces and proteins are salt precipitated.

In summary, H plasmids were isolated by all three methods. The Kado and Liu method yielded unacceptable amounts of plasmid DNA. The sarkosyl lysate method gave reasonable amounts of DNA, but was costly in that it required a great deal of CsCl. The Birnboim and Doly method was efficient both time and cost-wise, and gave suitable amounts of plasmid DNA for restriction enzyme

analysis. The appearance of a third band in the gradients obtained from this method does not affect the results from restriction enzyme digestion, and poses few problems for the use of the plasmid DNA obtained with this method. From 150mL of cells, the yields of DNA obtained with this method were comparable to those obtained with the sarkosyl lysate method prepared from 500 mL of culture. The reason for this is probably two fold. The Birnboim and Doly method uses overnight cultures, while the sarkosyl lysate method used mid-log cultures due to the capacity of the CsCl gradients. Secondly, the fact that the plasmid DNA is banded twice in the sarkosyl lysate method procedure results in a loss of CCC DNA.

The use of a protease treatment in the isolation of H plasmids appears to be of some significance, as noted in particular with the Kado and Liu isolation technique, in which there was a noticeable increase in the yield of CCC with protease treatment.

4.2 Restriction Enzyme Analysis

Restriction endonucleases are enzymes which recognize specific sequences within double stranded DNA. Type II restriction enzymes are useful tools for molecular biology. The recognition sequence for these enzymes is typically 4 to 6 nucleotides in length with a two fold axis of symmetry. The specificity therefore allows reproducible fragment patterns to be generated by a given enzyme for a given piece of DNA. The fragments can be resolved on the basis of molecular weight, by electrophoresis through an agarose or acrylamide gel system. The fragment pattern generated is called a restriction enzyme fingerprint as it is unique for each type of DNA. For fragments less than 1 kb in size, a polyacrlyamide gel system is used to resolve the fragments.

Restriction enzyme fingerprinting has been used to study the molecular relatedness of plasmids (Thompson et al, 1974). Previous molecular studies of H plasmids have revealed that there is little DNA sequence homology between the H plasmid subgroups. These studies gave an estimate of the percentage homology amongst these subgroups. More detailed studies including restriction enzyme digestion were undertaken in this study to confirm and extend these

earlier observations.

A diversity in DNA sequences amongst plasmids from the three HI subgroups was noted by comparing restriction enzyme digestions of a representative member of each subgroup. This was evident from the observation that there are no apparent common bands between all three subgroup plasmids. It was worth noting that there were trends in the frequency of cutting with a given enzyme. These trends in the frequency of cutting will be useful for further work such as restriction mapping of these plasmids. The H plasmids have been shown to have similar buoyant densities, and therefore similar G+C content (Whiteley & Taylor, manuscript in preparation), and this may account for the trends noted.

Within a subgroup, molecular relatedness was demonstrated by similar cleavage patterns. This was shown to be true for plasmids from both the HI1, and HI2 subgroups. In addition, despite the fact that the plasmids chosen in these experiments came from different geographical sources and hosts, they still demonstrated similar cleavage patterns. MIP233 is the only member of the HI3 subgroup, so that the major comparison was between HI1 and HI2 plasmids. The similar restriction enzyme patterns within a given subgroup may be useful as a diagnostic aid to assist in the assignment of an H plasmid to a given subgroup.

4.3 DNA Homology Studies

The methods used by Grindley *et al*, 1973, and Roussel and Chabbert, 1978 (see section 1.1[D]) in the studies of the molecular relatedness of plasmid incompatibility groups gave an estimate of the percent of DNA sequence homology between plasmids. With the advent of more advanced molecular biology techniques, more precise identification of DNA sequences showing homology are available through Southern transfer DNA hybridization techniques. Southern transfers are prepared from restriction enzyme digests in which the fragments are transferred to nitrocellulose membranes. The pattern of fragments is conserved in this transfer. The fragments are then probed with a 'nick-translated' plasmid for sequence homology such that the DNA sequences in common with the probe are identified on specific restriction fragments.

The DNA sequence homology amongst the H plasmid subgroups was investigated using these techniques, with a nick-translated HI1 plasmid as a probe. This was considered a natural extension of the restriction enzyme digestion experiments since it had been previously reported that a diversity in restriction enzyme patterns does not necessarily mean a lack of significant DNA homology

(Constantino et al, 1981). A number of restriction enzyme digests were transferred to nitrocellulose and examined for fragments showing homology. In general, the fragments showing homology were mainly the larger fragments produced from the digestion. The one exception to this of note was the digest of MIP233 with PstI (section 3.5).

The hybridization studies of Roussel and Chabbart, (1978), estimated the percentage of DNA homology amongst the H plasmid subgroups to be between 1 and 14%. In particular, HI1 and HI2 plasmids showed between 3 and 14% homology, and the HI3 plasmid, MIP233 showed between 1 and 6% homology with HI1 and HI2 plasmids. The use of Southern transfer DNA hybridizations for the quantitation of homology is difficult for several reasons and is therefore rarely used (Southern, 1981). For a given band showing homology it is not clear what percentage of this fragment is homologous with the probe. Furthermore, quantitation of this value based on the intensity of the band may be of some difficulty because large fragments may not transfer completely from the gel and film detection methods other than direct autoradiography do not give a linear response. The distribution and number of fragments showing homology varied with the enzyme used. The frequent

cutter, EcoRI showed the most number of fragments with some DNA homology with pRG1251 (between 7 and 10), whereas the infrequent cutter PstI gave the least number of these fragments (3-4). The error of interpretation of this technique with regard to the quantitative assessment of the percent of DNA homology is clearly demonstrated with the plasmid MIP233. In the case of this plasmid digested with EcoRI, the sum of the fragments showing homology with pRG1251 represents about 32% of the total DNA, and for the infrequent cutter PstI, this value is 4.7%. Rather it can be said that the PstI digest of MIP233 in which the fragments showing homology are minimized in the range of fragments of lower molecular weight (about 3kb in size) is about the best quantitative observation from these experiments concerning homology with this plasmid and pRG1251, although this is again an overestimation of the percent of homology within a given fragment. With the other digests of MIP233 this 4.7% homology is distributed throughout a number of fragments, some of which are in the upper molecular weight range. In the case of R478(HI2), a maximum estimate of the homology is between 29 and 38% when taking into account the total molecular weight of the fragments showing homology with

pRG1251. Again this is an overestimation of the percent of homology and appears to be large because the homology in this case is seen in the fragments in the upper molecular weight range. These fragments are much less intense than the fragments of pRG1251 hybridized against itself in the same molecular weight range, indicating that not all the DNA in the fragments of R478 in the upper molecular weight range is homologous to pRG1251 DNA.

The conditions under which hybridizations are performed determine the stability of the hybrids formed. These conditions determine the stringency of the reaction. This in turn is largely a function of the Tm (that temperature at which 50% of the helical structure of the DNA is lost) of the hybrids,which is affected by such parameters as salt conditions and formamide concentration in the reaction. The rate of reannealing of DNA is maximal at 20-25 degrees below the melting temperature (Tm), and hybridization is generally carried out under these conditions (Wetmur & Davidson, 1968). These conditions are considered to be quite stringent since for every 1% base mismatch between DNAs', the Tm is lowered by 1.4 degree C (Hyman et al, 1973). Therefore at a Tm-20° C one will detect only those hybrids in which there is 85% or greater base pairing.

Tm as determined as a function of formamide, salt concentration (Na+), and G+C content is determined by the following relationship (Howley et al, 1979).

$$Tm = 81.5 + 16.6(\log M) + 0.41(\%G+C) - 0.72(\%\text{formamide}) \quad (1)$$

where

M = monovalent salt concentration

%G+C = the percentage of guanine plus cytosine residues in the DNA

From this relationship, the higher the salt concentration, and formamide concentration, the lower the Tm.

The quantitation of the kinetics of hybridization of DNA immobilized on nitrocellulose filters is difficult to predict partly because the concentration of DNA bound to the filter and its availability for hybridization are unknown. The equations for these reactions have been described by Flavell et al (1974).

Howley et al (1979), studied hybridization of viral DNA immobilized on nitrocellulose filters under varying concentrations of formamide. Under stringent conditions (50% formamide, 37°C) five of six bases matched in a hybrid will be thermally stable. They determined that 19.2% + 2.6 of the SV40 genome was homologous to BK DNA, a result of which was in good agreement with previous findings that one could detect 20% homology between these

genomes under stringent conditions by solution hybridizations. Under less stringent conditions, (same temperature, less formamide) more homology was detected by this method of filter hybridizations.

Stringent washing conditions are close to the Tm of the duplex (these should be slightly below 5° C of the Tm of the hybrid in question (Maniatis et al., 1981)).

Using equation (1), the conditions for hybridization used in the experiments in this study are evaluated as follows: Hybridizations were performed with 5X SSC, 50% formamide. The Tm under these conditions is 64° C. The hybridizations were performed at 43° C, which is 21° C below the Tm. These conditions are therefore stringent.

The washing conditions for these hybridizations were performed in 0.1XSSC at 65° C. The Tm of the DNA under these conditions is determined using equation (1) at 73° C. The washing conditions were therefore at 8° C below the TM of the hybrids. Again these are stringent conditions.

The hybridization experiments performed by Roussel and Chabbert (1978) were also performed under stringent conditions. The hybridization conditions were in 50% formamide, 2X SSC at 37° C. The Tm under these

conditions as determined by equation (1) is 21 degrees C higher than the hybridization temperature. The washing conditions were equivalent to the hybridization conditions. The hybridization conditions from these studies match those of the Southern transfer DNA hybridization studies presented here, and therefore the fragments showing homology are those which were responsible for the homology observed by Roussel and Chabbert.

The conditions for hybridizations performed within a subgroup (HI1) were identical as those between subgroups. Extensive sequence homology was demonstrated amongst the group of HI1 plasmids used in this study. Based on this observation it is not unreasonable to assume that the HI2 plasmids which have similar cleavage patterns would also demonstrate extensive sequence homology, as would be predicted from the findings of Grindley and Roussel and Chabbert.

Table 21 lists the common phenotypic traits shared by the plasmids used in this study. It can be suggested that fragments showing homology contain some of the genes responsible for these traits. All three subgroup plasmids are incompatible with each other and the genes responsible for this may be amongst the fragments showing homology. pRG1251, and R478 both encode for resistance to tetracycline.

and chloramphenicol. All three subgroup plasmids produce H pili. MIP233 has no antibiotic resistances in common with pRG1251, and the homology noted with this plasmid as particularly pointed out with the PstI digest may be specifically involved in incompatibility or transfer functions. Finally, the restriction fragments showing homology with pRG1251 for the incHII plasmid may contain the genes primarily involved in pili production or some aspects of transfer since this plasmid is compatible with incHI plasmids.

H plasmids were initially divided into two groups based on their incompatibility reaction with F factors in the autonomous state. HI1 plasmids were incompatible with F factors, while H2 plasmids were compatible with F (see section 1.5). H1 and H2 plasmids have been further distinguished in that H1 plasmids have been primarily associated with chloramphenicol resistance in Salmonella typhi, while H2 plasmids were originally described in non-typhoidal strains of Salmonella and in Serratia marcescens, they have also been found to mediate antibiotic resistance in other Enterobacteriaceae. It would appear therefore that these plasmid subgroups have evolved separately in different genera which may account for the small amount of DNA homology between these groups.

Table 20

Common phenotypic traits shared
by the H plasmids used in this study

Plasmid	Incompatibility group	Resistance markers	H pilus production	Incompatibility with pRG1251
pRG1251	HI1	ApCmSmSpSuTc	+	+
R478	HI2	CmKmTcTe	+	+
MIP233	HI3	Te	+	+
PHH1508a	HII	(SmTp)[a],Te	+	-

a Resistances to Tp and Sm carried on Tn7(Bradley et al.,1982)

4.4 Concluding remarks

Successful isolation of H plasmids has allowed them to be characterized on a molecular level. For future studies, the Birnboim and Doly method of isolation allows simultaneous isolation of a number of H plasmids and by further scaling up this method, it should be now possible to isolate large quantities of H plasmid DNA.

These studies investigated the molecular relatedness of the H plasmids by restriction enzyme analysis and further by Southern transfer DNA hybridization techniques. Identification of the fragments showing DNA homology is useful to further characterize the H plasmid group. Presumably the restriction fragments contain some of the genes responsible for the incompatibility, replication and maintenance functions, or for transfer genes. Despite the large size of these plasmids the lack of DNA homology between the subgroups lends itself to identification of common genes. Cloning of these fragments and identification of the genes on these fragments would allow the nature of H plasmids to be better understood. The cloning of the small fragments of MIP233 showing homology with pRG1251 would be a good starting point for these studies.

In addition this study appears to be the first attempt at characterizing sequence homology between HI and HII plasmids.

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APPENDIX 1

Computer program for sizing DNA fragments

Estimation of the size of DNA fragments from agarose gels can be determined by several mathematical relationships. The size can be determined approximately by plotting the log of the molecular weight versus the distance migrated or the size may be plotted against the reciprocal of mobility(Southern,1979). Both of these relationships give a straight line for fragments of small molecular weight and curve in the large molecular weight range. Estimation of molecular weight by graphical means introduces unnecessary errors(Southern, 1979: Schaffer & Sederoff,1981).

Southern(1979) described a relationship based on the linearity of the plot of size versus the reciprocal of mobility which corrects for the curve in this plot in the upper molecular weight range to a straight line by plotting length(L) vs. $1/m-m_o$, where m=mobility and m_o is a correction factor which is described as follows:

If $L \propto 1/m$

then $L= k \times 1/m$ where k is a constant.

If the plot of L versus 1/m is a curve, then the measurements can be made to fit a line by the relationship:

$$L = k_1/(m-m_o) + k_2 \qquad (1)$$

where m_o is a correction factor such that three lines join three points with the same slope. A standard curve for this equation for three standard points with lengths L_1, L_2, and L_3 and mobilities m_1, m_2, and m_3 respectively can then be generated assuming that k_1 and k_2 are constants. m_o is then solved as

$$m_o = \frac{m_3(L_2-L_3)(m_2-m_1)-m_1(L_1-L_2)(m_3-m_2)}{(L_2-L_3)(m_2-m_1)-(L_1-L_2)(m_3-m_2)}$$

Finally, the values of k_1 and k_2 can be solved for given m_o ,using equation(1)

$$k_1 = \frac{L_1-L_2}{(m_1-m_o)(m_2-m_o)}$$

$$k_2 = L_1 - k_1(m_1-m_o)$$

Using this relationship the lenghts of unknowns can be predicted within the size range L_1 to L_3. This is a very useful relationship for measuring the sizes of molecules over a wide range of fragment sizes, as was the case with the restriction digests of the H plasmids. The computer program used in this study is based on this relationship except that it is expanded to include more than three points

for the standard, and it uses a least squares analysis of the equations. It would therefore account for the portion of the standard curve which deviates from linearity. The drawback of this program is that its accuracy is limited to the size range of the standards. This is of particular disadvantage with the infrequent cutters which generate fragments in the upper molecular weight range. This problem would however be present with other types of analysis.

Example 1.

Use of the computer program. The HindIII fragments of phage lambda are used as a standard curve, and the mobility of the EcoRI fragments of lambda are the unknowns.

```
$run *forts scards=len.dna spunch=obfile
$10:54:40
 MAIN        NO ERRORS
$10:54:41  T=0.152  RC=0
$run obfile 5=*source* 3=*sink*
$10:54:55

 # OF POINTS FOR STANDARD ? =  06
 ENTER MOLECULAR WEIGHTS   (8 PER LINE) ? =
 23.7,9.46,6.61,4.26,2.26,1.98,.58
 ENTER DISTANCES ? =
 52.0,70.5,81.5,96.5,126.5,133.5,

 STN LEN        DIST    FRED LEN        DEV          %        C(I)
     23.7      52.000      23.765     -0.065     -0.276     371.570
      9.5      70.500       9.363      0.097      1.023     375.710
      6.6      81.500       6.541      0.069      1.041     375.545
      4.3      96.500       4.389     -0.129     -3.022     364.920
      2.3     126.500       2.261     -0.001     -0.023     372.472
      2.0     133.500       1.955      0.025      1.247     374.887
 MO=      37.49976 LO=      -1.92506 CBAR=     372.51709
 SC=       4.08873 SD=       0.10886

 ENTER # OF POINTS FOR UNKNOWN ? =  06
 DISTANCES (8 PER LINE) ? =
 53.0,77.5,85.5,87.,92.5,106.5,
   FOR A DIST OF      53.0000  PREDICT A LEN OF      22.1079
   FOR A DIST OF      77.5000  PREDICT A LEN OF       7.3878
   FOR A DIST OF      85.5000  PREDICT A LEN OF       5.8357
   FOR A DIST OF      87.0000  PREDICT A LEN OF       5.6005
   FOR A DIST OF      92.5000  PREDICT A LEN OF       4.8479
   FOR A DIST OF     106.5000  PREDICT A LEN OF       3.4737

 # OF POINTS FOR STANDARD ? =
```

The sizes of the EcoRI fragments agree with the published values. (Table 6)

Computer program for sizing restriction fragments as adapted for use on the computer at the University of Alberta.

```
C LEAST SQUARES FIT OF DNA TO GEL MIGRATION
      DIMENSION WT(50), DIST(50), PROD(50), DWT(50), DDIST(50)
      DIMENSION DPROD(50), C(50), D(50)
      REAL*4 MO,LO,MWT,MDIST,MPROD
1000  CONTINUE
      WRITE(3,202)
202   FORMAT(/'&# OF POINTS FOR STANDARD ? =    ')
      READ(5,1) N
    1 FORMAT(I2)
      IF (N .LE. 0) GO TO 99
903   WRITE(3,203)
203    FORMAT(' ENTER MOLECULAR WEIGHTS   (8 PER LINE) ? =')
      READ(5,2,ERR=903) (WT(I),I=1,N)
    2 FORMAT(8F10.0)
904   WRITE(3,204)
204   FORMAT(' ENTER DISTANCES ? =   ')
      READ(5,2,ERR=904)(DIST(I),I=1,N)
      SWT=0.
      SDIST = 0.
      SPROD= 0.
      DO  11 I=1,N
      SWT=SWT + WT(I)
      SDIST = SDIST + DIST(I)
      PROD(I) = WT(I)*DIST(I)
   11 SPROD = SPROD + PROD(I)
      MWT = SWT/N
      MDIST = SDIST/N
      MPROD = SPROD/N
      DO 12 I=1,N
      DWT(I) = WT(I) - MWT
      DDIST(I) = DIST(I) - MDIST
   12 DPROD(I) = PROD(I)-MPROD
      CSSL = 0.
      CSSM = 0.
      CSCPML = 0.
      CSPMLL = 0.
      CSPMLM = 0.
      DO 13 I=1,N
      CSSL = CSSL + DWT(I)**2
      CSSM = CSSM + DDIST(I)**2
      CSCPML = CSCPML + DWT(I)*DDIST(I)
      CSPMLL= CSPMLL + DPROD(I)*DWT(I)
   13 CSPMLM = CSPMLM + DPROD(I)*DDIST(I)
      DET = CSSL*CSSM-CSCPML**2
      MO = (CSSM*CSPMLL - CSCPML*CSPMLM)/DET
      LO= (-CSCPML*CSPMLL +CSSL*CSPMLM)/DET
      SC = 0.
      SSC = 0.
      DO 14 I =1,N
      C(I)=(WT(I)-LO)*(DIST(I)-MO)
      SC = SC + (C(I) - C(1))
   14 SSC = SSC + (C(I)-C(1))**2
```

```
      SC = SC + (C(I) - C(1))
   14 SSC = SSC + (C(I)-C(1))**2
      CBAR = SC/N + C(1)
      SDC = SQRT((SSC - SC**2/N)/(N-1))
      WRITE(3,101)
  101 FORMAT('OSTN LEN',7X,'DIST',3X,'PRED LEN',8X,'DEV',10X,'%'
     X  ,7X,'C(I)')
      SD = 0.
      SSD = 0.
      DO 15 I = 1,N
      PREDWT = CBAR/(DIST(I)-MO) + LO
      WTDEV = WT(I) - PREDWT
      PERC = 100.*WTDEV/WT(I)
      SD = SD + WTDEV
      SSD=SSD+WTDEV**2
   15 WRITE(3,3) WT(I),DIST(I),PREDWT,WTDEV,PERC,C(I)
    3 FORMAT(' ',F8.1, 5F11.3)
      SDWT=SQRT((SSD - SD**2/N)/(N-3))
      WRITE(3,4) MO,LO,CBAR
    4 FORMAT(' MO= ',F12.5,' LO= ',F12.5,' CBAR= ',F12.5)
      WRITE(3,6) SDC,SDWT
    6 FORMAT(' SC= ',F12.5,' SD= ',F12.5)
      WRITE(3,205)
  205 FORMAT(/'&ENTER # OF POINTS FOR UNKNOWN ? =   ')
      READ(5,1)NU
      IF(NU.LE. 0) GO TO 199
  906 WRITE(3,206)
  206 FORMAT(' DISTANCES (8 PER LINE) ? =   ')
      READ(5,2,ERR=906)(D(I),I=1,NU)
      DO 16  I =1,NU
      PREDWT = CBAR/(D(I)  - MO) + LO
   16 WRITE(3,5) D(I),PREDWT
    5 FORMAT('   FOR A DIST OF ',F12.4,'  PREDICT A LEN OF',F12.4)
  199 GO TO 1000
   99 STOP
      END
le
```